IDENTIFICATION OF A NEW TARGET TISSUE FOR GROWTH HORMONE: THE PLACENTA

Izbel Yusuf

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Declaration

I, Izbel Yusuf, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Izbel Yusuf

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Abstract

Growth hormone variant (GH-V) is expressed in the human placenta. Placental GH-V is not produced in rodents, but pituitary GH production is increased in pregnancy. In both cases, pulsatile GH secretion is replaced by a more continuous GH pattern. The placenta also expresses GH receptors, so it itself could represent a target tissue for direct GH action. GH effects are mediated in part via the JAK/Stat5b pathway, and detection of phosphorylation and nuclear translocation of Stat5b can be used to identify cells responding directly to GH *in vivo*. I have now tested whether the rodent placenta shows Stat5b responses to GH *in vivo*. This has been studied under basal conditions, following continuous GH replacement, and during fasting, with or without insulin injection. Following a single iv injection of recombinant bovine GH (bGH) in female dwarf rats or GHRH-M2 GH-deficient mice, tissues were processed for phospho-Stat5 (pYStat5) immunocytochemistry. As expected, both non-pregnant and pregnant animals showed marked hepatic pYStat5 responses. Notably, pYStat5 responses were also seen in placental syncytiotrophoblast cells in response to injections of GH but not saline. GH exposure during pregnancy is continuous, and it has previously been shown in male dwarf rats that continuous GH exposure is much less effective in inducing hepatic Stat5 phosphorylation, and blunts the pYStat5 response to a GH pulse. Pregnant rats receiving continuous bGH, showed an increased cellular pYStat5 staining compared to saline controls. However, like in the male rats, the response to GH was blunted following continuous exposure. Fasting is known to impair GH effects, with lower pYStat5 responses in target tissues. GH-induced pYStat5 responses were reduced in fasted animals, a single injection of insulin was able to restore the response to GH during the continuing state of fasting, however, this was variable depending on the extent of endogenous GH. GH deficiency was also

associated with reduced litter size, average pup and placental weights. A preliminary microarray analysis was carried to identify potential GH target genes in the placenta. A few well known GH target genes or placental genes containing potential Stat5b response elements showed no change in response to GH or a change in secretory pattern. However, some novel genes were identified as GH-responsive and might be important in growth during pregnancy. In conclusion, my results show that the placenta is a direct target for GH, but what role this plays in placental function or fetal growth remains to be determined.

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Abbreviations

AKT	protein kinases
ALS	acid-labile subunit
bGH	bovine Growth Hormon
BMI	Basal Metabolic Index
BSA	Bovine Serum Albumin
$CaCL_2$	calcium chloride
cDNA	complementary Deoxyribonucleic Acid
CIS	cytokine inducible SH2-containing protein
CYP19	cytochromes P450
DC	detergent compatible
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GH	growth hormone
GHBP	growth hormone binding proteins
GHD	growth hormone deficient
GHRH	growth hormone releasing hormone
GHRH-R	growth hormone releasing hormone receptor
GHR	growth hormone receptor
GHS-R	growth hormone secretagogue receptor
GH-V	growth hormone variant
HCL	hydrochloric acid
hCS	chorionic somatomammotrophin
hCG	human chorionic gonadotropin

HDL high-density lipoprotein hPL

H_20_2 hydrogen peroxide

IGF-1 insulin like growth factor one

IGFBP insulin like growth factor binding protein

IGFBP insulin like growth factor binding protein

IGF-1R insulin like growth factor one receptor

IR insulin receptor

IRS-1, 2 and 3 insulin receptor substrates 1, 2 and 3

IUGR interunterine growth restriction

JAK2 janus kinase 2

Kb kilobase

Kg kilogram

LDL low-density lipoprotein

MBq megabecquerel

$MgCL_2$ magnesium chloride

NACL sodium chloride

NT non-transgenic

oPGH ovine placental growth hormone

PCR polymerase chain reaction

PFA paraformaldehyde

PI3ks phosphoinositide 3-kinases

PRL prolactin

Ras-MAP mitogen-activated protein kinase pathway

RIA radioimmunoassay

RNA ribonucleic acid

SDS	sodium dodecyl sulfate
SH2	src Homology 2 domain
SMS	sommatostatin
Stats	signal transducer and activator of transcription
SOCS	suppressor of cytokine signaling proteins
TSH	thyroid-stimulating hormone
WT	wild type

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1. Introduction

1. General Introduction

Growth hormone (GH) is the central endocrine regulator of postnatal growth and involved in the coordination of a wide range of biological processes. GH is also required for fetal nutrition and may have some indirect effects on fetal growth. The human growth hormone gene family is encoded within a 48-kilobase (kb) cluster on the long arm of chromosome 17. This cluster spans 66.5 kb and is made up of five genes, each of which is found within close proximity of each other [1]. The genes are highly related, owing probably entirely to gene duplications, having between 91% and 99% sequence homology [2]. The genes encode pituitary growth hormone (native GH, GH-N gene), placental growth hormone (GH variant, GH-V gene), and three chorionic somatomammotrophins (hCS-A, hCS-B and hCS-L genes), also called lactogens, [3]. The GH gene is approximately 3 kb long, consists of 5 exons and 4 introns, and encodes a 217-amino-acid precursor protein [2]. An amino-terminal signal peptide is subsequently removed by proteolytic cleavage yielding a mature single-chain polypeptide that contains 191 amino acids with a molecular mass of 22-kDa [4, 5]. A 20-kDa form of GH has also been found to be secreted by the pituitary and is produced by alternative splicing of the GH precursor mRNA [6].

Genes encoding rat and mouse GH have been shown to be situated on chromosome 10 [7] and 11 [8] of the rat and mouse genomes respectively. Mouse GH is encoded by a single copy gene located in a highly conserved region between mouse chromosome 11 and human chromosome 17. Other genes from the PRL/GH family, including the genes encoding for mouse PRL, and placental lactogens have all been mapped to chromosome 13 [8]. Like in the mouse and in humans, PRL genes are located on different chromosomes, with rat PRL located on chromosome 17 [7].

1.1 GH, regulation, action and secretion.

Pituitary GH is known to be under neuronal, hormonal and metabolic control. GH is synthesized and secreted by the somatotroph cells in the anterior lobe of the pituitary gland [2, 4]. GH secretion from somatotrophs is a calcium dependent event, during which an increase in cytosolic calcium is required for the release of GH [9]. GH synthesis and secretion is regulated by two hypothalamic peptides, growth hormone releasing hormone (GHRH) [10] and somatostatin (SMS) [11], via their opposing effects on intracellular calcium concentration [12, 13]. GHRH elicits an increase in intracellular calcium via its G-protein-coupled receptor, in part by stimulating an increase in the level of cellular cAMP [14]. SMS has an opposing effect on intracellular calcium concentration and inhibits GH synthesis and secretion [14].

A class of molecules known as GH-releasing peptides [15] also stimulates GH release. The GH-releasing peptides are a group of short synthetic peptides that stimulate GH release via binding to the GH secretagogue receptor (GHS-R) [16]. The GHS-R cDNA encodes a seven-transmembrane G protein-coupled receptor of 364 amino acids and is highly conserved between rats, human, and pig [16]. Kojima *et al* (1999) [17] identified and purified the endogenous GHS-R ligand termed ghrelin, a

28-amino-acid peptide that can stimulates the release of GH both *in vitro* and *in vivo*. The identification of ghrelin whose primary source is the stomach, suggests that GH release can be regulated by peripheral signals, as well as of those generated by the hypothalamus. GH exerts its biological actions by binding to a specific cell surface receptor, the growth hormone receptor (GHR). The GHR belongs to the class I cytokine receptor superfamily that includes receptors for PRL, erythropoietin, leptin, interferons, granulocyte colony stimulating factor, and the interleukins [18]. These receptors are singlepass transmembrane proteins that contain an extracellular region, a single hydrophobic transmembrane domain of 24 amino acids, and an intracellular signaling region. The human GHR (hGHR) gene is localized on the short arm of chromosome 5 in the region p13.1-p12 [19].

The mouse GHR (mGHR) gene is similar in size and sequence to the hGHR gene but contains two additional exons [20]. These include an exon 4B, which is downstream of exon 4, and an exon 8A, which is upstream of exon 8. Exon 4B encodes an eight-amino-acid segment of the extracellular domain of the receptor and is present in all known mGHR transcripts. Exon 8A serves as an alternative splice site for the mGHR and rat GHR gene [20, 21], to generate a soluble binding protein. This is also present in humans, and is derived by proteolytic processing of the extracellular domain of the human GHR. The GHRs are a single polypeptide chain that range from 614 – 626 amino acids in length with a predicted molecular mass of approximately 70 kDa [22, 23]. The extracellular region of GHR contains seven cysteine residues and five potential N-linked glycosylation sites that are highly conserved between species, [23]. GHRs are present in many biological tissues and cell types, including liver, bone, kidney, adipose, muscle, eye, brain, and heart [24]. GHRs have also been identified in

immune cells including cultured human B cells [25] IM-9 lymphocytes [26], spleen, and thymus [27].Extensive work has been done to characterize the signaling pathways activated by GH-N [28, 29], and recent years have shown significant progress in elucidating the signaling pathways activated by the interaction of GH and GHR. An initiating event in this interaction is the activation of janus kinase 2 (JAK2), a GH receptor-associated tyrosine kinase [30].

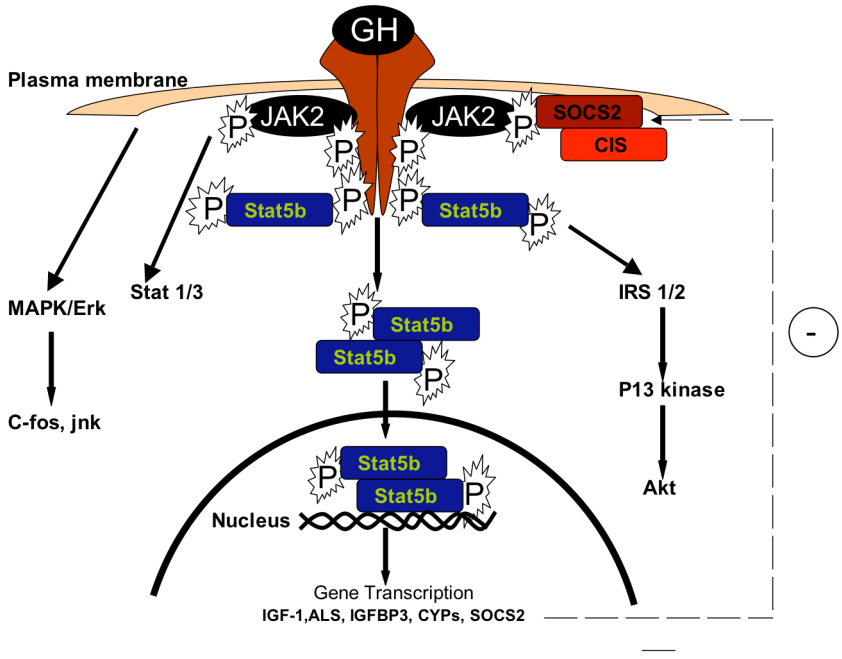
The original model of GH activation of JAK2 was the binding of GH to two GH receptors to induce dimer formation, which would lead to the increase in affinity of each GH-receptor for JAK2 [31, 32]. However, Waters *et al* (2006) [33] revised this original concept, using FRET based studies to show that GHRs already exist as dimers. It is thought that GH binds to these dimers causing rotation of one of the GH-receptors, which results in GHR-JAK2 association, bringing JAK2 molecules into close proximity, leading to the phosphorylaton of the activating tyrosine sites on each JAK2 molecule, locking JAK2 in an active confirmation [34]. It is thought that activated JAK2 then phosphorylates itself and the cytoplasmic domain of the GH receptor on tyrosine residues [29]. These tyrosines are thought to form high-affinity binding sites for a variety of signaling proteins that contain phosphotyrosine binding domains, such as the signal transducers and activators of transcription (Stat) family of transcription factors. Activated GH-JAK2 can phosphorylate at least four members of this family (Stats 1,3,5A and 5B), leading to their dimerization, nuclear translocation, DNA binding and activation of transcription [35].

The most important member of this family is Stat5, as has been shown with targeted disruption of Stat5a and Stat5b genes. A study by Udy *et al* (1997) [36] evaluated the importance of Stat5b in the physiological effects of GH pulse using a mouse gene

knockout model. The disruption of the Stat5b gene led to the loss of multiple, sexually differentiated responses associated with the sexually dimorporhic pattern of GH secretion. Both male characteristics of body growth rates and male-specific liver gene expression were decreased to wild-type female levels. The findings of this study and other *in vivo* studies [37] suggest that Stat5b may be the major Stat protein that mediates the sexually dimorphic effects of GH pulses in the liver. This is further emphasized with the highly homologous Stat5a being unable to substitute for Stat5b [38]. An elegant study by Rowland *et al* (2005) [39] reported knock-in mouse models that express GHRs with deletions of specific intracellular domains of the receptor. These mice displayed progressive impairment of postnatal growth, furthermore, Stat5 phosphorylation was substantially decreased for some of the mutant models and absent from others, correlating with loss of IGF-1 expression and reduction in growth. They also showed however, that an additional pathway important in GH regulated gene transcription is the mitogen-activated protein kinase pathway (Ras-MAP). The Ras-MAP kinase pathway has also been shown to initiate GH regulated fos gene transcription [40, 41]. The GH-JAK2 activated complex also interacts with other pathways activated by other hormones such as insulin and IGF-1. GH is known to have both insulin-like and anti-insulin-like effects. The insulin-like actions include, glucose and amino acid transport, lipogenesis and protein synthesis. GH and insulin are thought to converge on common signaling pathways, as GH stimulates the phosphorylation of the insulin receptor substrates 1, 2 and 3 (IRS-1, 2 and 3), (Fig 1.1).

Important regulators of GHR-JAK2 signaling are a family of cytokine-inducible genes, termed suppressors of cytokine signaling (SOCS), and cytokine inducible subunit (CIS) [42]. GH induces the expression of eight members of the SOCS family, SOCS 1, 2, 3 and CIS in the rat liver [43]. SOCS proteins are thought to inhibit signaling by binding to JAK2 directly [44]. Transgenic mice constitutively expressing CIS have reduced body weights [45] presumably because of decreased GH-induced activation of JAK2. Mice deficient in SOCS2 however, are giant, suggesting that SOCS2 might be important for terminating signaling by GH or IGF-1 [46].

Figure (1.1) Growth hormone activated signal transduction cascades

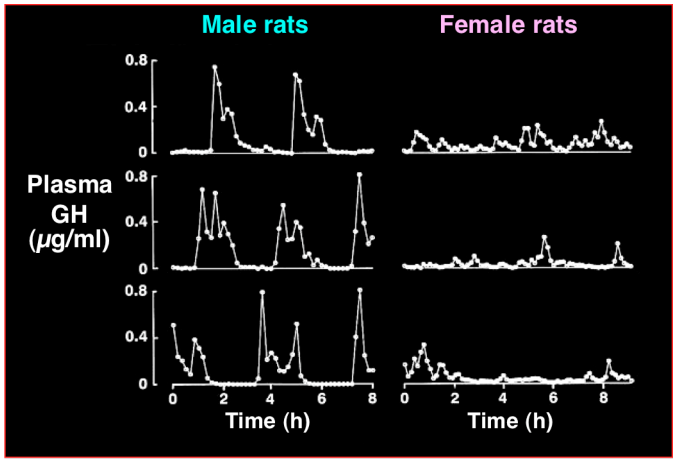


1.2 GH secretory patterns

The secretion pattern of pituitary GH is characterized by multiple episodic bursts commonly referred to as GH pulses [47]. However, a notable sex difference in the pattern of GH secretion is evident in most mammalian species [48] and [49]. This is particularly striking in rodents; secretory profiles recorded in male rats are shown to be characterized by high-amplitude GH bursts at regular 3- to 4-hr intervals, separated by prolonged (1-2hr) periods of mostly undetectable plasma GH levels [47].

In contrast, female rats exhibit irregular, more frequent, lower amplitude GH pulses superimposed on an elevated GH baseline [50], (Figure 1.2). These distinct sex differences in the temporal pattern of GH release are of biological significance, as they evoke a number of male-female differences in, (i) body growth, (ii) GH intracellular signaling pathways, and (iii) liver enzyme expression.

Figure (1.2) GH secretory profiles for normal male and female rats



An automatic method for repetitive microsampling of blood from conscious animals was used to obtain detailed GH-secretory profiles, for the first time, Clark et al., 1987.

1.2.1 GH secretion pattern and Body Growth

Early studies by Tanner (1963) [51] and Eden (1979) [52] have shown that body weight and composition begin to differ in male and female rats from around the onset of puberty. The sex differences in the pulsatile profile of circulating GH, along with the amplitude of GH, is likely to be the major contributing factor to the differences shown in the growth of rodents [53]. Male rats infused with GH at a constant rate show steady increases in growth rate and circulating IGF-1 levels, however, in the same rats, a constant GH infusion was shown to be more effective in increasing hepatic GH-binding protein concentrations, than infusion of the same GH dose as a series of pulses [50, 54-56]. In contrast, pulsatile infusion is much more effective in promoting statural growth [57]. Gevers *et al* (1996) [78] confirmed that growth responses in the rat are more sensitive to stimulation by the pulsatile component of a GH infusion, whereas hepatic GH-receptor and plasma GH-binding protein concentrations are more sensitive to a continuous component. This and other studies point to the greater amplitude of intermittent GH pulses in male rats, as most effective component in stimulating growth in GHD animals than the type of profile observed in female rats. Note however, that females do grow in response to continuous GH, just less rapidly. If female rats are given GH pulses they will grow more like males.

There is also a sexually dimorphic pattern of GH secretion in humans: mean 24 –hr serum concentrations of GH are significantly higher in females, than in males [58]. In a study by Pincus *et al* (1996) [59] blood sampling in a small cohort of young women and men showed that the females exhibited an increased irregularity in their GH pulsatility, compared to males. These alterations in the pattern of GH signal in humans, is thought to have similar effects on body composition as shown in rodents.

Furthermore, Hindmarsh *et al* (1997) [60] demonstrated that peak and trough GH concentrations have different associations with the IGF axis, body composition and metabolic parameters in adult males. They concluded that peak values of GH concentration profiles were mostly responsible for influencing the IGF axis, whereas trough values may affect body composition and metabolic parameters of GH action.

1.2.2 GH secretory pattern and intracellular signaling

In the adult male mouse or rat, GH pulses stimulate an intracellular signaling pathway that is dependent on JAK2, and Stat5b [36, 61]. This sex-dependent plasma GH pattern to activate Stat5b determines distinct, sex-dependent patterns of gene expression in the liver (discussed below). From studies in hypophysectomized rats, the importance of the time interval between each plasma GH pulse has also been highlighted [50, 62, 63]. The time between each pulse is critical in determining the ability of liver cells to reset their signaling systems to respond to subsequent GH pulses. A study by Waxman *et al* (1991) [64] further emphasized the ideas of a minimum GH "off time" for hepatocytes to express the masculinizing effects of pulsatile GH. Hypophysectomized rats given rat GH subcutaneously, required at least 2.5 hours between each interpulse trough to give rise to distinct patterns of male liver gene expression. These findings suggest that male rat hepatocytes may become refactory to subsequent GH pulses until a minimum recovery period has lapsed.

Furthermore, an *in vitro* study carried out by Gebert *et al* (1999) [65], using CWSV-1 liver cell lines, showed that the level of Stat5b tyrosine phosphorylation was reduced to 10-20% of the maximal GH pulse-induced Stat5b signal, within 3 hours of continuous GH exposure. Thus, GH-responsive cells reduce their response following

continuous GHR occupancy. Several studies have also shown that the targeted disruption of Stat5b leads to major loss of multiple, sexually differentiated responses associated with pulsatile GH secretion [36, 66]. Thus, GH signaling is critically impaired in animals with Stat5b disruption, and thus has been confirmed by the observation of severe short stature in patients with Stat5b mutations [67, 68]. A study carried out in hypophysectomized Stat5b deficient mice, showed no changes in the body weight, following GH pulse replacement, compared to wild-type mice, which showed dramatic resumption of body weight [69]. Furthermore, a study by Klove *et al* (2007) [70] presented evidence to show the importance of skeletal muscle Stat5 in postnatal growth and suggested that this is conveyed by the production of localized IGF-I. To investigate the role of Stat5 in skeletal muscle, mice with skeletal muscle specific deletions of both combined Stat5a and Stat5b genes (Stat5MKO) were used. These mice showed a reduction of 60% in IGF-I mRNA levels in muscle tissue. At 8 weeks despite a reduction of only 15% in circulating IGF-1 levels, mice showed a 20% reduction in body weight, accounted for by a reduction in lean mass. Finally, the skeletons of the Stat5MKO mice were also found to be smaller than controls, indicating that the defect was not restricted to skeletal muscle alone. These findings provides strong evidence that Stat5b has a role in mediating body growth, stimulated by the male pattern of pulsatile plasma GH.

1.2.3 Sexually dimorphic liver expression

The sex dependent plasma GH pattern has been shown to lead to sex-dependent patterns of gene expression in the liver, the major target of GH action [71]. This is exemplified by the patterns of transcription of sex-dependent steroid hydroxylase P450 genes. The male intermittent pattern of GH secretion stimulates the expression

of male specific steroid 16α- and 2α-hydroxylase P450 CYP2C11 [64, 72, 73], while the female continuous plasma GH profiles up regulate steroid disulfate 15β-hydroxylase P450 CYP2C12 [74]. This suggests that GH can initiate intracellular signaling pathways via the GH-receptor, with very different opposite effects on some targets, which are determined by the temporal pattern of plasma GH stimulation.

Studies in hypophysectomized rats given tailored patterns of GH have further confirmed that steroid metabolism in the liver can show feminization or masculinization depending on the mode of GH administration [75]. Furthermore, Wells *et al* (1994) [76] showed that sex differences in steroid-metabolizing enzymes could still be found in dwarf rats with low GH secretion, as it still remains dimorphic, small pulses in males and low continuous GH levels in females. In this study, male dwarf and normal rats given continuous intravenous infusion of very low doses of human GH, showed a dose-dependent decrease in male specific cytochrome P450s, and an increase in female specific cytochrome P450s, with no effect on IGF-1 and minimal effects on growth. This shows the high sensitivity of hepatic cytochrome P450 transcripts to GH. The correlation between Stat5b tyrosine phoshorylation and GH pulses, have also implicated in directly determining the expression of male-specific liver genes, with the finding that some GH pulse-regulated, male specific liver P450 genes contain Stat5b response elements, for example the CYP3A10 gene [77].

The sexually dimorphic pattern of GH secretion has also been shown to be important in functions other than steroid metabolism, some of these include, GH- binding protein expression in the liver [78] and major urinary protein secretion [79], suggesting that many GH targets are sensitive to the pattern of GH. The significance of this for my work is that there are two conditions in humans in which the normal pulsatility is converted to a high continuous physiological GH secretion, namely fasting and pregnancy. In fasting, this high GH exposure is accompanied by a desensitization to the effects of GH, with low IGF-1 levels. In pregnancy however, this is not accompanied by fasting, so I predict it is likely to be related to increase GH signaling. Modeling fasting in rodents doesn't help as fasting reduces GH secretion in rodents (unlike in humans) [80]. However rodent pregnancy is also associated with increased GH secretion [81]. I felt it was therefore an excellent model to study the *physiological* relevance of continuous GH.

1.3 Actions of GH, GH-V, IGF-I and Insulin

1.3.1 Role of GH

GH physiological role is pleiotropic and affects several tissues including, bone, liver, fat and muscle. As its name implies, a major role of GH is to stimulate longitudinal bone growth, and growth of other tissues, actions of GH are summarized in Table 1.1. GH directly stimulates prechondrocytes in the growth plate followed by a clonal expansion caused both by the GH-induced local production of IGF-1 and by a GH-induced increase in circulating levels of IGF-1 [82]. During the process of longitudinal bone growth, prechondrocytes in the germinal cell layer differentiate and thereafter undergo limited clonal expansion in individual chondrocyte columns in the growth plate [83]. Several hormones are necessary for the expansion of normal

postnatal longitudinal bone growth, but it is generally accepted that GH is the most important stimulatory hormone in this respect. GH stimulates growth of cartilage and other tissues by both increasing the number of cells and by increasing cell size [84], though the latter may also include an indirect effect via IGF-1.

Table (1.1) Biological actions of GH

Stimulates	**Inhibits**
Longitudinal bone growth &	Insulin action
Bone remodeling	IGFBP-1 synthesis
Chondrocyte proliferation	IGFBP-2 synthesis
Osteoblast proliferation and bone deposition	
Osteoclast proliferation and bone reabsorption	
Type I collagen synthesis	
Skeletal muscle growth (fiber, strength)	
Liver growth	
Deiodination of T4 to T3	
Lipolysis	
Ketogenesis	
Gluconeogenesis	
Protein synthesis/turnover	
Total body nitrogen balance	
Lactation	
IGF-I synthesis	
ALS synthesis	
IGFBP-3 synthesis	
Serine protease inhibitors (SPI) 2.1 and 2.2	
Immunomodulation	
(endocrine, autocrine-paracrine)	
B and T cell proliferation	
Natural Killer cell activity	
Macrophage activity	
Neutrophil activity	
Immunoglobulin production	
Cytokine production	

Adapted from Le Roith et al., 2001

A widely discussed question during the last two decades has been whether GH acts on tissues directly, or whether the effect is mediated by a liver-derived growth factor, initially called sulfation factor [85], later renamed somatomedin, and subsequently shown to be identical to IGF-1. According to the original somatomedin hypothesis, GH stimulates skeletal growth by stimulating liver production of somatomedin, which in turn, stimulates longitudinal bone growth in an endocrine manner [86]. This was because in early studies it was very difficult to demonstrate any effects of GH *in vitro*.

However, in the early 1980s the somatomedin hypothesis was challenged by a landmark study demonstrating that injection of GH directly into the rat tibia growth plate stimulated longitudinal bone growth at the site of injection [87], in one leg, but not in the control injected leg. This initial observation has subsequently been confirmed and extended, and it is now well documented that GH stimulates growth of many different tissues directly [88, 89]. However in agreement with the original somatomedin hypothesis, IGF-I- null mice fail to respond to GH treatment, suggesting that IGF-I is essential for GH-stimulated postnatal growth. However, experiments carried out in the liver specific IGF-1 deficient (LID) mice [90] showed that normal growth and development could proceed despite low circulating IGF-1 levels, suggesting that other mechanisms besides hepatic production of IGF-I may be involved in growth. Although 75% of circulating IGF-1 is liver-derived, normal growth and development is possible even in the complete absence of liver IGF-1 production [91]. Taken together, these studies allow to hypothesize that GH mediates somatic growth both directly and via local IGF-1 production, acting in a paracine/autocrine fashion in different tissues. Liu *et al* (2000) [92] treated LID mice with exogenous GH and studied postnatal growth (in response to long-term injections)

and extra hepatic IGF-1 expression (following acute injections). They demonstrated that recombinant human GH stimulated local tissue IGF-1 production under conditions of complete liver IGF-1 deficiency, particularly so in adipose tissue. These results provide further support to the notion that exogenous GH accelerates somatic growth through stimulation of local production of IGF-1, as well as by direct mechanisms, via a Stat5b phosphorylation.

The importance of GH in longitudinal bone growth and bone formation is obvious in isolated GH deficiency, in which bone mass and length is also reduced [93]. However, the low IGF-1 levels caused by GHD, will also contribute to the reduction in bone mass. Again, these studies emphases that whilst GH plays a major role in the longitudinal bone growth an inter-relationship with IGF-1 is evident and difficult to disentangle. To reconcile this, a dual theory concerning the roles of both GH and IGF-1 was suggested, in which, GH stimulates the specific differentiation of cell types, while IGF-1 stimulates their clonal expansion, [94]. However, there have also been disputes of the dual action theory for GH and IGF-1. Although GH has been confirmed to have direct effects on the differentiation of growth plate germinal cells, the further proliferative effect of IGF-1 on chondrocytes has been disputed [83]. Both Shiner *et al* (1993) [95] and Wang *et al* (1999) [96] were unable to detect IGF-1 mRNA in the growth plate chondrocyte of rats and mice of any age. Nevertheless, IGF-1 clearly has an important role in human and rodent longitudinal bone growth, since IGF-1 gene deletions result in dwarfism in mice and short stature in humans [97, 98].

Furthermore, IGF-1 treatment of children with Larons syndrome produces an increase in bone growth, demonstrating that IGF-1 can work independently of GH, as GHR is inactive in this syndrome [99].

The availability of GH to the tissues is also modulated by growth hormone binding proteins (GHBPs). Growth hormone binding proteins were first detected in the serum of pregnant mice by [100] and were then reported in human serum [101, 102]. Following the cloning of the GH-receptor, an identity between GHBPs and GHR was demonstrated [22, 103]. GHBP in humans originates by specific proteolysis of the GH receptor [104]. However, in rodents the GHBP is produced by alternate messenger RNA splicing of the eighth exon and contains a unique hydrophilic carboxy terminus [105]. The concentration of GHBP in serum has been reported to be higher in females than in males and is oestrogen- dependent. This is the case in humans [103, 106], and rats [107-109]. Since the GH binding domain of the high affinity GHBP is identical to GH binding domain on the membrane associated GHR, the binding of GH to GHBP could reduce its availability for interaction with the GHR, and it has been calculated that at low GH levels, as much as 30-50% of GH circulates coupled to the GHBP [110]. However in practice it is a much smaller proportion, when measured directly by half-life measurements [111] of larger amounts of GH.

Pregnancy in rodents is the only physiological state in which there is a dramatic change in serum GHBP concentration. An upregulation was initially observed in mice by Peters (1977) [100] and subsequently by Smith (1988) [112] suggesting a role for GHBP in normal pregnancy. Furthermore, Sanchez *et al* (1990) [113] showed that hypophysectomy of mice on day 11 of gestation resulted in down regulation of GHBP

mRNA and circulating GHBP. The mechanism is obscure but may relate to induction of estrogen [114]. In contrast, in humans, although there is a several fold increase in maternal GH concentration from week 18 onwards [115], after an initial increase in GHBP concentration, they decrease. Such a concomitant decrease in GHBP would tend to increase the proportion of bioavailable GH for GHR signaling, but this has not been directly demonstrated in humans.

GH is not just a hormone for growth. It also has several important metabolic effects in adulthood, a major one being the induction of protein synthesis in muscle tissue, which in turn is also responsible for the regulatory control of the mobilization of nitrogen reserves, used to provide further amino acids to various organs [116-118]. GH enhances the uptake of amino acids into skeletal muscle. The importance of this effect of GH is evident in GH-deficient individuals, who show a marked reduction of lean body mass and skeletal muscle mass, compared to healthy individuals [119, 120], whilst GH replacement therapy results in the increase of muscle mass [121, 122]. GHD adults receiving GH replacement increase their total lean body mass by as much as 11% [123] and thigh muscle mass by 5-8% [124, 125]. Again it is possible that some of the effects of systemic GH on muscle protein metabolism may also be a result of increasing circulating and / or local IGF-1 production.

In this context, several studies have looked at the acute and long-term actions of GH administration on whole body metabolism, and have demonstrated that GH has direct effects on increasing anabolic actions via the inhibition of amino acid oxidation and stimulation of whole-body protein syntheses [126]. Systemic GH may enhance local production of IGF-1 and IGFBP-4 in muscle. IGF-1 is shown to stimulate satellite

myoblasts to express myogenin, which mediates the differentiation of myoblasts to myotubes and to mature myocytes [127-129]. It is also possible that GH may *directly* increase the total number of myocytes or may stimulate mature myocytes to increase autocrine expression of IGF-1.

GH also has important lipolytic effects. It enhances the utilization of fat by stimulating lipolysis and fat oxidation [130]. GH brings about its lipolytic action by inhibiting lipoprotein lipase, an enzyme involved in lipid accumulation in adipocytes [131, 132] and represents a major effect of GH on metabolic intermediates. GH exhibits both insulin-like and insulin antagonizing actions on both glucose and lipid metabolism in adipose tissue [133]. The insulin like effect is an acute antilipolytic and lipogenic effect, causing a temporary insulin-like effect on stimulating glucose uptake. This acute insulin-like activity of GH on carbohydrate metabolism seen both *in vivo* and *in vitro* appears to be independent of both circulating IGF-I and insulin, since these effects have also been observed in isolated tissue preparations and in cultured cells [134]. It may therefore represent another direct effect of GH.

Insulin-*antagonizing* effect of GH include inhibition of lipogenesis and glucose transport, and the increase of lipoysis [132], which predominates with chronic GH treatment. GH administration also causes mild reductions in low-density lipoprotein (LDL) cholesterol levels and small elevations in high-density lipoprotein (HDL) cholesterol [135]. The clinical significance of these effects is reflected in the increased adiposity in GH deficiency and reduced fat mass in acromegaly [136, 137] and in GHD individuals given GH replacement therapy. Studies in animal models of GH deficiency models have also recorded increased fat mass, and that treatment with

GH decreases adipose mass, and increases increased lean body mass [138]. In obese dwarf rats, GH treatment induces a dose-regime-dependent effect on lipolysis, with GH in the presence of IGF-1 showing anti insulin actions causing a powerful net lipolysis [139]. Chronic exposure to GH, however, showed insulin resistance associated with hyperinsulinemia that seems primarily due to a post receptor defect in insulin signaling [140].

1.3.2 Effects of GH-excess and GH-deficiency in insulin signaling

In both humans and animals an excess of GH causes the impairment of insulin sensitivity. This results in glucose intolerance, insulin resistance and hyperinsulinemia [133, 141-143]. In contrast, GH deficiency leads to increased insulin sensitivity, decreased secretion of insulin and hypoglycemia [144, 145].

The mechanism of this cross-talk between GH and insulin signaling pathways is unclear, but both *in vitro* and *in vivo* studies show GH promotes tyrosine phosphorylation of IRS-1 and IRS-2 and their association with PI 3-kinase [146] [147-149]. These results suggest a direct way for GH to interact with signaling molecules used by insulin. Such potential crosstalk between the GH and insulin signaling pathways is further emphasized in studies carried out in GHR-knock out mice. A study by Dominici *et al* (2000) [150] showed that the absence of GHR was associated with increased IR abundance, insulin-stimulated IR tyrosine phosphorylation, and normal efficiency in IRS-1 phosphorylation, and activation of PI-3 kinase by insulin. Furthermore, a more recent study of Dominici *et al* (2002) [151] looked at the effects of combined GH, PRL, and thyrotrophin (TSH) deficiency on insulin signaling in Ames dwarf mice. These showed metabolic alterations associated with a change in insulin signaling, increased protein levels of IR, IRS-1

and IRS-2, insulin-stimulated association of p85 with IRS-1 and IRS-2, and increased insulin induced AKT activation through a PI3-kinase independent mechanism. These findings suggest that the deficiency of GH, PRL, and TSH is associated with increased insulin sensitivity, Furthermore, metabolic alterations are also associated with changes in insulin signaling. However, whether these compensatory changes in insulin signaling have an important role in the phenotype of Ames mice is not entirely clear [150]. Interestingly, these animals also live much longer than their normal siblings [152] and paradoxically reductions in insulin signaling (or in homologous pathways in other species) have been associated with increased longevity [153].

1.3.3 The effects of nutritional restriction on GH and Insulin signaling

As mentioned above, Growth hormone secretion is affected by fasting differently in different mammalian species [80, 154-156]. In humans, nutritional deprivation is associated with an *increase* of circulating GH [157] and a decrease in IGF-1[158]. The increase in GH secretion is thought to be linked to an increase in pituitary sensitivity to GHRH and a decrease in pituitary sensitivity to somatostatin. In contrast to humans, fasted rats show a dramatic *decline* in pulsatile GH release [80, 159]. This decrease is thought to be associated with the decrease of hypothalamic GHRH expression [159, 160]. However, consistent with humans, IGF-1 levels decrease in food deprived rats [161]. Furthermore, fasting is also shown to induce some form of GH resistance, a study by Beauloye *et al* (2002) [162], showed the reduced IGF-1 levels were not restored by GH administration. This state of GH resistance is thought to be associated with the decrease of GHRs, as evident by reduced GH binding [163, 164]. The consequence of this is the possible impairment of the JAK-STAT signaling pathway, in concert with induction of suppressors of cytokine signaling (SOCS),

particular SOCS3 [162]. Unfortunately however, the very different responses to fasting between rodents and humans, makes it difficult to study mechanisms relevant to GH and human fasting, in rodent models.

1.4 IGF family

The insulin-like growth factors (IGFs) are integral components of multiple systems controlling both growth and metabolism. The IGF family consists of two ligands (IGF-1 and IGF-2), six major and several minor IGF binding proteins, and cell surface receptors that mediate the actions of the ligands (IGF-1 receptor and the IGF-2 mannose-6-phosphate receptor) and the insulin receptors which can be activated to some extent by IGFs [165, 166]. In circulation, almost all the IGFs are present as 150 kDa ternary complexes comprising of one molecule each of IGF, IGF-binding protein- (IGFBP)-3 (the predominant IGFBP in serum) or to a lesser degree IGFBP-5 [167, 168] and an 85-kDa glycoprotein, the acid-labile subunit (ALS) [169, 170].

ALS is primarily a plasma protein, which serves as a reservoir of systemic IGFs by sequesting the majority of these within ternary complexes from which the IGF is slowly released, principally after limited proteolysis of IGFBP-3 within the complex [167, 171]. The ALS complex may also enhance long-term body growth by maximizing the bioavailability of systemic IGFs. Short-term studies using IGF-1 [172, 173] were carried out in GH-deficient animals, but all components of the ternary complex are decreased in GHD, making it difficult to outline the separate roles of ALS, IGF-I and IGFBP-3. An ALS-null mouse model has been generated, in which the ternary complexes were absent due to the inactivation of the ALS gene [174]. Null ALS mice show dramatically reduced circulating IGF-I and IGFBP-3 concentrations compared with their wild-type siblings (62 and 88% reductions respectively). These

changes occurred despite the absence of any reductions in IGF-I or IGFBP-3 synthesis, as expression of both these genes in liver, the predominant site of synthesis, was normal, proving ALS is necessary for the normal serum levels of both IGF-I and IGFBP-3 [175, 176]. However, despite disturbances in the circulating IGF system, null ALS animals show only a 13% growth reduction by adulthood [177-179]. This is consistent with the observation that abrogation of IGF-I synthesis only in liver, resulting in a reduction of plasma IGF-1 similar to that of the null ALS mice, does not alter postnatal growth [90, 91]. However, double gene disruption of IGF-1 and ALS by crossing IGF-1 deficient (LID) and ALS knockout mice resulted in further reductions in serum IGF-1 levels and a significant reduction in linear growth, suggesting that some threshold concentration of circulating IGF-1 is necessary for normal bone growth [180] to occur. Again, interpretation is complex, since with low circulatory IGF-1, negative feedback is reduced and GH levels become very high. IGFs bound to the 50 kDa binary complexes, can cross the vascular endothelium, but formation of the ternary complexes restricts the IGFs to the circulation, prolongs their half-lives and allows them to be stored at high concentration in plasma, facilitating their endocrine actions. It may also help to minimize the effects of intrinsic insulin-like activities, such as hypoglycaemia [172].

1.4.1 IGF-1 physiological role in growth and metabolism

IGF-1 has an important role in both embryonic and postnatal growth. This is demonstrated in mouse models carrying null mutations in the IGF-1 gene, which are born small, compared to wild-type and continue to grow poorly postnatally [97] [177, 181]. Furthermore, infusion with IGF-1 will enhance body weight and length, showing that circulating IGF-I can stimulate growth [182].

IGF-1 has insulin-like metabolic actions as well as unique actions of its own. A unique action is displayed in its involvement in the increase of protein synthesis as well as inhibition of proteolysis [183]. However, as insulin acts primarily to inhibit proteolysis, these findings also suggest that IGF-1 works via the IGF-1 receptor and not the insulin receptor in the muscle. An obvious insulin-like action of IGF-1 is the enhancement of glucose uptake in peripheral tissue [184]. Furthermore, insulin-like effects of IGF-1 replacement have been shown with patients with type 1 and type-2 diabetes. Plasma glucose concentrations in these patients decrease following acute or chronic administration of IGF-1 [185, 186]. Similar findings with a decrease in glucose levels in conditions of insulin resistance and mutations of insulin receptor has also been reported following short term IGF-1 treatment [187-189]. The mitogenic and anabolic actions of IGF-1 are believed to be mainly mediated by signaling through the IGF-1 receptor [190]. The IGF-1 receptor shares high degree of homology with the insulin receptor, both receptors having the same heterotrameric structure formed from two α- and two -β subunits; the homology extends to 85% in the cytosplasmic tyrosine kinase domain, which is thought to initiate intracellular events involved with receptor signaling, [191].

1.4.2 Pathological conditions of IGF-1

IGF-1 is important for intrauterine growth, as IGF-1 and IGF-1 receptor knockout mice show a reduction in birth weight of 45%, compared to normal pups [97, 177, 181]. The effects of IGF-1 are mediated by the IGF-1R, knockout mice for IGF-IR and IGF-1 show a reduction in weight (40% of normal) [97]. IGF-1 gene deletions and mutations also have compromising effects in humans. IGF-1 deletion results in severe intrauterine growth restriction and postnatal problems in growth [98].

Furthermore, low IGF-1 levels in cases of IGF-1 promotor region polymorphisms, are associated with a reduction in birth weight and length [192, 193].

1.4.3 Insulin and Insulin signaling

Insulin induces a wide range of growth and metabolic responses, a major role is its regulation of glucose, lipid and protein metabolism in known GH target organs including liver, muscle and fat [194]. Insulin signaling begins with the binding of insulin to the α-subunit of the insulin receptor (IR), a protein tyrosine kinase. This initiates the autophosphorylation of tyrosine residues in the intracellular β-subunit region of the insulin receptor, and several intracellular proteins, including insulin receptor substrate (IRS)-1 and IRS-2 [195]. These substrates are crucial in coordinating the effects of insulin, [196-198] and IR substrates also provide docking sites for several SH2 (scr homology 2) domain-containing proteins. SH2 domains typically bind phosphorylated tyrosine residues, linking IR to other intracellular signaling cascades [195], such as phosphatidylinositol (PI) 3-kinase, a well-characterized SH2 domain-containing protein. Following the tyrosine phosphorylation of IRS-1 and IRS-2, binding of these proteins to the p85 regulatory region of P1 3-kinase occurs, resulting in the activation of the enzyme [199]. A downstream target for P1 3-kinase is protein kinase B (Akt), Akt is activated by phospholipid binding and phosphorylation at two regulatory sites [200], and is thought to mediate many insulin responses, such as insulin induced glucose uptake and glycogen synthase activation [201].

1.5 Growth Hormone Variant

Over 20 years ago the discovery of the growth hormone variant (GHV) meant a new addition was made to the human growth hormone gene family making a total of five, [202]. The discovery identified a "new" gene called the growth hormone variant (GH-V) the interest of this thesis, is that this GH-V has a unique source of production in the placenta, [115].

1.5.1 The GHV gene, its expression, protein product and measurement

The GH- V gene, yields an 800-nucleotide mRNA [203] and a 1250- nucleotide mRNA resulting from alternative splicing [204]. The 800-nucleotide mRNA codes for the 22 kDa GHV, GHV contains the same total number of amino acids as GH-N but differs at 13 positions and, moreover, exists in a minor, glycosylated form [205] [206]. Specific expression of the GH-V gene in the placenta has been demonstrated by *in situ* hybridization [207] and by immuno-histochemical localization [208]. More recently, GH-V gene expression has also been demonstrated in the extravillous cytotrophoblast, which invades the uterus in early pregnancy, as well as choriocarcinoma cell lines [209, 210]. Notably, the expression of GH-V mRNA was observed in a patchy appearance, i.e. in only some of the syncytiotrophoblastic cells, from as earlier as 9 weeks of gestation [207, 211] GH-V has somatogenic activity (at least in rodents), equal to GH-N [212-214]. Furthermore, transgenic mouse lines expressing hybrid gene, mouse metallothionein-1 (MT)/human placental GH variant display greater increases in adult body weight compared to normal mice [215] showing systemic effects of this GH-variant. Thus, the human placenta produces an active GH isoform.

1.5.2 Measuring Placental Growth Hormone

In the absence of a specific antibody for GH-V, the initial method used to measure growth hormone variant was performed indirectly using a GH immunoassays, both based on monoclonal antibodies (MAbs); one recognizing both the placental and pituitary GH form (MAb 5B4), and the other recognizing only the pituitary GH form (MAb K24). By subtracting the paired hormone concentration values obtained from each assay in each serum sample, the difference remaining was assumed to represent the placental form of GH in serum samples [216]. However, this method was indirect, as the assay couldn't distinguish directly between GH-N and GH-V and other GH isoforms. More accurate, specific assays have since been developed. The first assay developed to determine specific GH-V serum levels was based on a solid-phase immunoradiometric assay. This assay used non-glycosylated 22kDa recombinant GH-V expressed from *Escherichia coli* as a calibrator, and to determine the total amount of GH-V, mouse MAbs were used [217]. However, more recently this assay has been replaced by an enzyme-linked immunosorbent assay (Biocode) where its manufacturers claim that little cross-reactivity is seen from either 20-22kDa GH-N, human placental lactogens, prolactin or human chorionic gonadotropin (hCG). In 2004, the Strasburger group developed a non-isotopic GH-V assay, using mouse MAbs raised against the glycosylated variant of GH-V [218].

1.5.3 Secretion pattern, action and regulation of GH-V

Serum profiles of growth GH-V have been recorded for 24 hours in women at different stages of normal pregnancy [219]. In this study, two monoclonal antibodies directed against different epitopes, unaffected by human placental lactogen were used in radioimmunoassays to distinguish the pituitary 22kDa-GH-N from the placental

GH variant. GH-V was detected as early as 5 weeks of pregnancy. From there on GH-V levels gradually increased to peak values of around 25μg/L at week 34-37 of gestation. These are significant quantities of GH, not unlike those seen in mild acromegaly. The episodic peak activity of GH-N in non-pregnant and first trimester pregnant women was shown to dramatically reduce into a more continuous and stable secretion during late pregnancy, this change first observed at 17 weeks gestation. This study concluded that during the second half of pregnancy, serum measurements of GH reflect a major contribution from a non-episodically secreted placental GH variant and a concomitant suppression of pituitary GH [219, 220]. Other studies have also reported the non-pulsatile secretion of GH-V and how it dominates in the third trimester of pregnancy, down regulating and suppressing the pituitary source of GH, [221, 222]. Upon delivery of the placenta, the source of GH-V disappears and the serum concentrations start to decline immediately, with a reported half-life of 13 minutes [223]. In the course of pregnancy, serum GHBP levels follow a bell shaped curve. Increasing levels are seen in the first half of pregnancy, and then a decline is observed [224]. It is assumed that GH-V will bind to GHBP, which may facilitate accumulation in the circulation. Plasma GH levels have also been measured in female rats during late pregnancy. An early study, by Sauders *et al* (1976) [225] measured GH levels in rats during the last week of pregnancy. GH was measured in blood samples collected via chronic intra-atrial cannulae, every 15 minutes. The results indicated that there was an increase in GH levels in the last 3-4 days of pregnancy. GH levels have also been determined in late pregnant rats by Klindt *et al* (1981) [226]. Blood samples were collected over a 4-h period at 15-min intervals on days 18 and 19 of gestation. From days 18-19, GH levels increased 2-fold [226].

Plasma GH patterns have been also analyzed more extensively using a pulse analysis computer program (PULSAR), to analyze data from automated repetitive blood sampling from conscious pregnant animals. Mean GH levels were about twofold higher in pregnant females on days 15, 18 and 22 of gestation than in age-matched non-pregnant females [81]. The basal plasma GH levels were also increased, while there was no change in GH pulse amplitude or frequency. The augmentation of GH release was even more pronounced on day 20 of gestation, with a fourfold increase in mean plasma GH levels compared with those in non-pregnant females. This increase reflected an increase in both basal plasma GH levels and GH pulse amplitude, but there was no increase in pulse frequency. In female rats that delivered on day 22 of gestation, the basal and mean plasma GH levels increased during parturition. This is the most complete study in rodents to date and demonstrates an increase in basal plasma GH levels during late pregnancy, and a marked increase in both basal plasma GH levels and GH pulse amplitude on day 20 of gestation. Since hypophysectomy of the pregnant rats results in undetectable GH levels, the high levels of GH during rat pregnancy arise definitely from the pituitary source [81], and that there is no rodent placental source of GH-N, nor any evidence for a rodent GH-V.

To date there has not been a specific GH-V receptor identified that may mediate the actions of GH-V. However, GH-V displays very similar characteristics, including a high affinity for hepatic GH receptors, as GH-N [227]. Therefore, it is reasonable to think that GH-V may also act through the same GHR as GH-N. In *situ* hybridization and northern blot hybridization using complementary radioactive DNA probe encoding part of the extracellular domain of the GHR has been carried out. GHR gene was expressed in all human tissues studied, including the placenta [228]. Evidence for

the expression of the GH receptor (GHR) gene in the placenta was also obtained by northern blot analysis by a study by Frankenne *et al* (1992) [229]. GH receptor have been detected as early as 8 weeks gestation in syncytial layers of the placenta and shown to be maintained until term [230]. In addition, Frankenne *et al* (1987) [227] have detected GHR poly A+ RNA in RNA from cultured trophoblastic cells, but not from placenta fibroblasts. There was a low but significant specific binding of pituitary GH-N and placental GH-V to placenta plasma membranes. Both variants were shown to bind to the same receptor, which is present in the first trimester as well as in the term placenta. These early findings suggest the provocative notion that in humans GH-V may have paracrine or autocrine functions in the placenta as well as potential endocrine functions in maternal blood stream. GHRs have also been detected as early as 15 weeks in the syncytiotrophoblast cells of rat placenta. Receptor expression was localized by immunohistochemistry with specific monoclonal antibodies in several tissues including decidual and trophoblastic cells of the placenta [231]. Furthermore, a study by Ymer *et al* (1989) [232] also identifies binding sites for growth hormone in both fetal and maternal compartments of rat placenta. All these studies again suggest the idea that GH may have a role in placental metabolism in the rodent.

1.5.4 Regulation of GH-V

GHRH, which stimulates GH-N synthesis and secretion in the pituitary somatotrophs, is mainly expressed in the hypothalamus. However it is also expressed in extrahypothalamic sites, such as the placenta. In particular, it has been reported that the GHRH gene is actively transcribed in rat [233] and mouse [234] placentae. In both species, expression of the GHRH gene in placenta is regulated during gestation, increasing from mid- pregnancy to term [235]. The placental GHRH transcript and its

peptide products appear to have the same size as their hypothalamic counterparts, while the site of its placental GHRH synthesis is the cytotrophoblast [236, 237]. However, administration of GHRH to pregnant women does not to alter GH-V concentrations. Furthermore, addition of GHRH to the media of cultured trophoblastic cells from early and term human placenta, did not stimulate GH-V release [238]. Since that GHRH does not affect GH-V secretion, the physiological role of placental GHRH during pregnancy thus remains unclear [239]. Meigan *et al* (1988) [240] detected no immunoreactive GHRH in the rat maternal circulation suggesting that placental GHRH does not affect the maternal hypothalamic pituitary axis. The presence of high molecular weight immunoreactive GHRH in rat placenta but not in the median eminence also suggests that posttranslational processing of the GHRH precursor molecule may be different in the two organs [240]. Several studies suggest that glucose levels may play a part in regulating GH-V production. *In vitro* studies have shown that low glucose concentrations in culture media can lead to an increased liberation of GH-V from cultured placental explants [241]. More recent studies report an inverse relationship of maternal BMI and GH-V serum levels, implicating metabolic factors could possibly regulate GH-V [242, 243].

1.5.5 Physiological effects of GH-V

In rodents GH-V is a more "somatogenic" hormone as it has the same potency in binding to GHR as does GH-N, but has lower lactogenic bioactivity [213]. The ability of GHV to bind to somatogen or lactogen receptors was investigated in rabbit and rat liver microsome cell lines [214]. GH-V was found to displace ^{125}I-ovine prolactin bound to rat liver microsomes (lactogen binding) and to displace ^{125}I-hGH bound to rabbit liver microsomes (somatogen binding). Therefore, human GH-V is

predicted to display both somatogenic and lactogenic bioactivity in animals, a dual specificity previously thought to be unique to GH-N. However, a 7.4-fold difference in this ratio was observed for GH-V compared to GH-N, suggesting significantly greater selectivity by GH-V in binding to the rabbit somatogenic receptor. MacLeod *et al* (1991) [213] also showed that GH-V could bind to both somatogen and lactogen cell surface receptors *in vitro* and that the ratio of its somatogen to lactogen receptor-binding affinities was substantially higher than that of GH-N.

The somatogen bioactivity of GH-V was also assayed by stimulation of weight gain in hypophysectomized rats, and lactogen bioactivity was assayed by the mitogenic response of the Nb2 lymphoma cell line [213]. The average increase in rat body weight in response to a fixed concentration of hormone was comparable using either GH-V or GH-N, whereas the mitotic response of the lactogen-inducible Nb2 cells was significantly less for GH-V. The comparable somatogenic, but lower lactogen, bioactivity of GH-V relative to GH-N parallels the previously reported receptor binding profiles of the two hormones. While this can suggest that GH-V could have the potential to perform a unique role during human gestation, it is difficult to extrapolate because the specificities of the receptors for rodent and human GH isoforms differ. For example Goodman *et al* (1981) [244] investigated the biological properties of GH-N and GH-V. GH-N and GH-V activities were examined on rat adipocytes or epididymal fat segments. Both GH-N and GH-V were quite similar in their ability to bind specifically to intact fat cells and were virtually indistinguishable in their ability to increase glucose oxidation (an insulin-like response), induce refractoriness to insulin-like stimulation, and induce lipolysis in the presence of glucocorticoid. These findings suggest that placentally expressed GH-V has a

spectrum of metabolic activity comparable to pituitary GH-N and may contribute to regulation of carbohydrate and lipid metabolism during pregnancy. Some studies of GH-V have used human GHR-expressing cells for example, Silva *et al* (2002) [245] investigated GH-V signaling in IM-9 human lymphocytes. Human placental lactogen did not activate signaling in these GH receptor-expressing cells. Moreover, like GH, signaling by GH-V was inhibited by the GH antagonist (G120K). These findings confirm that GH-V can activate target cells expressing human GH receptors.

1.5.6 Physiological effects of GH-V during pregnancy

The direct function, if any, of GH-V during pregnancy is unclear, however, its continuous secretion appears to have important implication in the control of maternal IGF-1 levels. Studies in normal and pathological pregnancies have shown that IGF-1 values in the maternal plasma correlate with corresponding GH-V values, regardless of complications and gestational age. The Cauifriez group [222, 246] obtained blood samples from 93 healthy pregnant women at various gestational stages. IGF-1 and human placental lactogen (hPL) was measured by radioimmunoassay. Pituitary GH was estimated by a two monoclonal antibody-based radioimmunoassay, the K24 assay, which recognizes only GH-N, and the 5B4 assay, which reacts with all known pituitary as well as placental GH variants. GH-V was then distinguished from the main pituitary GH through its differential immunoreactivity. The results obtained showed that the mean plasma IGF-1 levels remained relatively stable until 29-30 weeks of gestation, and then began to progressively increase from 164.0±44.6μg/L to a maximum of 331.6±63.7μg/L by 35-36 weeks. Regardless of gestational age, IGF-1 values exhibited a positive correlation with GH-V values, whereas no significant correlation was found between IGF-I and hPL.

The regulation of IGF-1 with GH-V is also apparent in a study of pregnant women with acromegaly. Despite the high levels of GH-N and high basal IGF-1 concentrations observed, maternal IGF-1 levels were nevertheless shown to increase further during pregnancy, following the pattern of GH-V secretion [247]. Moreover, the description of progressive elevations of IGF-1 in pregnant PIT-1 deficient women (and hence pituitary GHD women) further supports the theory of GH-V as being a prime regulator of IGF-1 during pregnancy [248]. The actions of GH-V are most likely to be mediated indirectly by IGF-1, since GH-V does not cross the human maternal/fetal barrier [239]. Pregnant rats injected with ^{125}I-hGH hormone on gestational day 20 also showed no detection of radioactivity in fetal tissue, confirming no transfer of hGH from mother to fetus in the rat. The involvement of local IGF-1 production in fetal development was suggested from early studies [249, 250], with evidence that the secretion of IGFs from maternal decidua may play a role in the control of growth process. However, clear mechanisms have never been shown. A role for human IGF is supported by studies showing a direct correlation of maternal IGF-1 and birth weight [251, 252].

Further support for the coupled importance of GH-V and IGF-1 maternal concentrations come from studies that show decreased levels of both GH-V and IGF-1 in cases of intrauterine growth retardation (IUGR) [221, 253]. Chowen *et al* (1996) [254] evaluated GHV mRNA expression in placenta taken from normal and IUGR babies. There was a significant decrease GH-V mRNA expression in placentae of babies with IUGR compared to normal placentae. They suggests that in IUGR the decreased levels of GH-V in the maternal circulation may not result exclusively from the reduced size of the placenta, but also from abnormal placental tissue development and/or from abnormal regulation of GH-V synthesis. In addition, data from Larcoix *et*

al (2002) [255] has demonstrated the expression of GH-V in invasive extravillous trophoblasts suggesting that the physiological role of GH-V might also include a direct influence on placental development via an autocrine or paracrine mechanism [255].

1.5.7 GH-V in pathology

GH-V levels have been shown not to differ in the maternal circulation in cases of fetal anencephaly, supporting the independence of GH-V regulation from the fetal pituitary axis [253]. Women with a total deletion of the CS-A-B-GH-V gene locus (also resulting in the absence of circulating hPL) show uncomplicated pregnancies with normal newborns [256]. A further two reports of children with a GH-N, GH-V, CS-A and CS-L gene major deletion have also been published [257, 258]. The first of the cases lack accurate documentation, the second study, however, reported that the four affected newborns were short. These cases of deletion provide some evidence that the viability of human fetuses is not crucially affected by the lack of placental synthesis of GH, but this could contribute to defective fetal or placental growth

Other studies have pointed to a possible modification of GH-V expression in diabetic pregnancies. A study by Hu *et al*. (1999) observed a higher GH-V/CS-L mRNA ratio compared to normal term placenta [259]. Furthermore, McIntyre *et al*. (2000) [224] showed a strong correlation between GH-V and glycemia at 28–30 weeks of gestation and they suggest that in long-term regulation, GH-V levels in diabetic pregnancy may be driving increased glycemia [224]. Although rodents show no GH-V-like activity, it has been reported in other species like the sheep placenta [260]. In this study the ovine trophoblast and syncitium was shown to produce an ovine placental growth hormone (oPGH). This hormone is produced between day 30 and 60 of pregnancy,

peaking at day 55. As well as the production of oPGH in sheep placenta, GH receptors were also found to be expressed in sheep placental trophectoderm [261]. Golos *et al* (1993) [262] showed that Southern blots of rhesus genomic DNA probed with a human CS cDNA demonstrated mRNA levels of mCS1 and mGH-V, being most abundant and increasing from the first to second trimester and then remaining relatively constant.

1.5.8 Relationship of GH-V with Human Placental Lactogen (hPL)

Human placental lactogen (hPL), is a single polypeptide of 191 amino acids also called human chorionic somatomammotropin and the product of the two chorionic somatomammotrophin genes (hCS-A, hCS-B) [263]. hPL is related to the pituitary growth hormone and prolactin with which it shows 85% and approximately 30% amino acid sequence homology, respectively [264]. hPL is also synthesized by the syncytiotrophoblast of the human placenta [265] and is detected from an early stage of pregnancy. hPL has been shown to act in concert with GH-V in the stimulation of IGF-1 production, this result in the increase in the availability of glucose and amino acids to the fetus [266, 267]. A study by Pilistine *et al* (1984) [268] demonstrated that the administration of hPL to fasted pregnant rats increased maternal and fetal plasma IGF concentrations and stimulated fetal weight gain. Such findings lead to the proposal that PL could play a part in the regulation of IGF during pregnancy. A correlation has also been shown between birth weight and maternal levels of hPL [269]. Pregnant rats infused with hPL between days 14 and 21 showed a significant increase in fetal weight on day 19 of gestation [270]. Furthermore, Spellacy *et al*. (1976) [271] have shown that in comparison with normal pregnancy, women with complications of IUGR during pregnancy have shown significantly lower hPL values,

which positively correlates with both placental and fetal weights. With these findings, as well as hPL being secreted into both the maternal and fetal circulations after the sixth week of pregnancy [267], hPL may have direct as well as indirect effects on the fetal growth. Furthermore, Freemark and Cormer (1989) [272] documented a unique placental lactogen receptor in maternal and fetal sheep liver. These studies strongly suggest that the biological actions of placental lactogen in fetal tissues are mediated through binding of the hormone to a distinct and unique placental lactogen receptor.

Although hPL levels measured in maternal serum follow a similar pattern to GH-V and serum levels are measured to be 200-300 fold higher, two studies have now compared the relationship of GH-V, hPL and IGF-1, and hPL was shown to be only weakly associated with the third trimester IGF-1 values [222, 246]. Although hPL may be involved in the adaption to pregnancy, however, its potency over GH-V to regulate IGF-1 production is less, as the affinity of hPL GHR is >2000 fold weaker than for GH, whereas it has for binding to PRL-R [273]. Although hPL bears similarities to GH-V in placental production and implications in IGF-I regulation, the receptor binding properties imply different roles for hPL. There additionally seems to be no relationship between high or low glucose concentrations and hPL production, as shown for GH-V [241].

1.6 The placenta

The survival and growth of a fetus is crucially dependent on the placenta, as it forms an interface between the maternal and fetal circulation, facilitating metabolic and gas exchange as well as fetal waste disposal. In addition, the placenta produces hormones that alter maternal physiology during pregnancy and forms a barrier against the maternal immune system [274]. The number of hormones produced by the placenta, vary between species. In both humans and rodents, the fully developed placenta consists of three distinctive layers, the outer maternal layer, which includes decidual cells of the uterus as well as the maternal vasculature, which brings blood to and from the implantation site. A middle region, known as the junctional zone in rodents only, which attaches the fetal placenta to the uterus and contains fetoplacental (trophoblast) cells, that invades the uterine wall and maternal blood vessels. Lastly, an inner layer is composed of highly branched villi that are designed for efficient nutrient exchange [275].

Structural homologies and dissimilarities between mouse and human placenta are fairly well recognized. Although the gross architecture of the human and mouse placenta differ, their overall structure and the molecular mechanisms underlying placental development are thought to be similar [275]. Comparison of placental development in mouse and human is summarized in Table (1.2).

Table (1.2) Comparison of pregnancy and placentation in mouse and human

	MOUSE	**HUMAN**
Implantation	Secondarily interstitial	Primarily interstitial
Trophoblast invasion of uterine arteries	Shallow, limited to proximal decidua	Extensive: reaching myometrial vessels
Transformation of uterine arteries	Dependent on maternal factors (uterine natural killer cells)	Dependent on trophoblast
Placental exchange area	Labyrinthine	Villous
Trophospongium	Extensive	Absent
Interhaemal layer	Three trophoblast layers; outer one cellular, inner two syncytial	Single layer of syncytial trophoblast (Langhans layer not part of barrier)
Placental hormones	Placental Lactogens	Chorionic gonadotrophin (hCG), chorionic somatomammotrophin (hPL), placental growth hormone (GH-V), Progesterone, Relaxin, IGFs.
Gestation	Three weeks	Nine months

Adapted from Hans and Carter, 2000

1.7 Placental cell lines

A number of placental lines have been used to investigate several aspects of placental function. There are three main types of cell lines derived from human placenta; those that have arisen from spontaneously from cultured cytotrophoblast *in vitro*, those that have been immortalized by *in vitro* transfections with viral genes, and those from spontaneous choriocarcinomas, which have been maintained *in vitro* [276]. Defining the cell lines as representative of trophoblastic cell types has come from reports showing the production of hCG and hPL from cell lines [277, 278].

Spontaneous cell lines of placental origin have had some assessment of endocrine function [279]. Transfected cell lines on the other hand have been generated by specific transfection with viral gene, all of which are positive for at least one of hCG or hPL [280]. The drawback of transfected cell lines is that the alteration to the genome may result in an alteration of endocrine function, rendering them as less useful models for functional studies [276]. BeWo and Jar1 are well established human cell lines, derived from choriocarcinoma which are malignant tumors of epithelial origin, they are thought to be representative of trophoblastic cells, and shown to undergo fusion and morphological differentiation similar to the formation of syncytiotrophoblast by the cytotrophoblast in the placenta [281]. There is evidence to suggest that chorionic somatomamotrophin (hCS-A and hCS-B) and growth hormone variant gene expression, although relatively low, occur in BeWo cells, with relative expression is greater in BeWo cells than Jar-1 cells [282]. Treatment of BeWo cells with thyroid hormone results in a 6-fold increase in messenger RNA from placental members of the hGH gene family [283]. Studies have been carried out to compare chorocarcinoma cell lines compared to one another, to gain insight into how normal trophoblast cells might function. These have more similarities then differences, suggesting that these can be good models of the human trophoblast lineage [284] There are some differences however. For example Mandl *et al* (2002) [285] showed a difference in proliferative response to insulin and IGF-I in BeWo, Jar-1, and JEG-3 cell lines.

1.8 Aims of the thesis

Although human placenta makes growth hormone GH and its receptor GHR, but the physiological roles and potential interaction are based on circumstantial evidence. Now we have tools for studying direct effects of GH on tissues, the main aim of my thesis was to investigate whether the placenta could be shown to be direct target of growth hormone. I began by initially investigating the production of GH-V and expression of GHR in placental cell lines, with the hope of establishing a system I could use to study GH-V storage and regulation. However I mostly wanted to study an *in vivo* model in GHD to obtain direct evidence for the placenta as a direct target of GH.

To see an effect of GH, *in vivo* I used the approaches of giving GH to GHD animals. There were a number of GH-deficient animal models that were available to me for this project, including a *dw*/*dw* dwarf rat, as well as the GH-deficient GRF-M2 transgenic mouse. The advantage of using GH deficient animal models is to observe the direct effect of GH administration in a number of tissues without the interference of endogenous pituitary GH. A notable change that occurs during human pregnancy is the change in GH secretory pattern from the pituitary. This would be eliminated in a GH-deficient model, whilst I could administer different patterns of GH in pregnant GHD animals. I planned experiments to observe the effects of food deprivation on GH response under conditions of minimal endogenous GH alteration, and compared them with responses in normal animal models.

When I began my work I was promised mice from Carole Mendelson whose group produced a transgenic mouse (for other purposes) with human growth hormone expressed in the placenta. The objectives of this group were to investigate the placenta specific transcriptional factors conserved between humans and rodents. They introduced the 501 bp of CYP19 exon I.1 5' flanking sequence, which mediates placenta specific expression of aromatase, and used human growth hormone simply as a reporter in their transgenic mice. Expression of the CYP19 (I.1):hGH fusion genes was shown to be placenta specific. I envisaged I could examine placental responses to endogenous (transgene) hGH in isolation, by crossing these mice with our GHD mice strains, which would then lack pituitary GH, generating the first mouse model to have only a placental source of GH. Unfortunately, due to a variety of circumstances (loss of mice, huricanes and floods) these mice never arrived. I therefore decided to concentrate on GH administration studies, and used both rat and mice GHD models instead. To compensate, I also performed some preliminary studies of potential GH gene targets in placenta using microarray, to investigate preliminary downstream targets of GH in the placenta.

In summary, the objectives of this study is to (i) investigate whether the placenta is a direct target of GH, (ii) whether the secretory pattern of GH and other factors i.e. restriction of nutrition alters the response to GH, and finally (iii) to seek preliminary evidence for potential downstream targets responsive to GH and sensitive to GH secretory pattern in the placenta.

Materials and Methods

2. Materials & Methods

2.1 Animal physiology experiments

2.1.1 Animal models

GRF-M2 transgenic mice were generated by Paul Le Tissier. These mice have their GHRH neurons ablated using a modified H37A variant of the influenza virus and M2 protein [286]. The mice show GH deficiency, secondary to GHRH deficiency, and develop dwarfism following weaning.

Dwarf rats (*dw/dw*) were originally characterized by Charlton *et al* (1988) [300] but since have been bred as a homozygous colony in the SPF unit at NIMR. The dwarf rat mutation is autosomal recessive, and arose spontaneously in a breeding colony of Lewis wild-type rats. The mutation has not been identified, but causes a profound, but not total specific GH deficiency, with over 90% reduction compared to normal AS rats.

2.1.2 Animal stocks

Mice were bred and housed in the Laidlaw Blue animal unit of the NIMR, maintained according to local and national ethical animal welfare guidelines with a 12hr light/12hr dark cycle. Unless otherwise stated animals were allowed free access to a standard chow diet (3.4% fat, 18.8% protein, 3.7% fibre, 3.8% ash and 60% carbohydrate). Tap water was provided as drinking water. Animals were housed usually, in groups of 2-4. On completion of experiments, animals were anaesthetised using isoflurane, weighed, and then decapitated to collect truncal blood. Blood was collected in tubes containing 50U of heparin (Leo Laboratories Ltd), centrifuged at

200g for 10 minutes and plasma supernatant aliquoted into 0.5mL microfuge tubes and stored at -20^{o}C until further use. Tissues were collected, weighed, frozen on dry ice and stored at -80^{o}C or fixed where appropriate in 4% paraformaldehyde (Sigma) for 16 hours before being transferred to 70% ethanol.

2.2 Genotyping

To identify transgenic pups within litters, ear punches were taken from 2-week-old mice and DNA isolated for genotyping. Each sample was digested overnight in 250µL lysis buffer (50mM Tris pH 8.0, 100mM NaCl [Sigma-Aldrich], 100mM EDTA, 1% SDS [Bio-Rad], in dH_2O) containing 0.4mg/mL proteinase K (Roche) at 55^{o}C. DNA was extracted by adding 80µL of saturated NaCl solution to precipitate proteins and cell debris by centrifugation, followed by the addition of isopropanol (Fisher Scientific) to precipitate DNA and washed in 70% ethanol (Fischer Scientific) to remove salt. DNA was resuspended in 50µL water (Molecular biology grade, Sigma) and kept at 4^{o}C until further use.

2.2.1 Polymerase Chain Reaction (PCR)

Amplification of DNA fragments was performed by PCR. DNA (1-2µL) was amplified by using 40 cycles of denaturation, annealing and extension conditions of 94^{o}C/40 seconds, 58^{o}C/40 seconds, 72^{o}C/120 seconds. A total reaction volume of 25µL was used, consisting of 200µM dNTP mix (GE Healthcare), 0.2µM primers, 1x reaction buffer IV (ABgene), 1.5mM $MgCl_2$ (ABgene), 2U Taq Polymerase (ABgene).

Table 2.1 lists the primers used to genotype GRF-M2 mice, and are directed against specific 3' and 5' sequences of human GH in the GRF-M2 transgene, which provides upstream cloning sites in GHRH and a polyA site, Le Tissier *et al* (2005) [286].

Table (2.1) Oligonucleotides used to genotype transgenic mice.

PRIMER	DIRECTION	SEQUENCE
GH12	hGH 5' UTR	AACCACTCAGGGTCCTGTGGACAG
GH13	hGH 3' UTR	ATGATGCAACTTAATTTTATTAGGACAA

Products were separated using electrophoresis through a 1.2 % agarose gel in 1x TBE (10x stock: 89mM Tris-Base, 89mM boric acid, 2mM EDTA) with 5ng/mL ethidium bromide (Bio-Rad) for analysis. The product sizes expected for non-transgenic (NT) and transgenic (T) were 1523bp and 724bp, respectively.

2.3 Animal treatments

2.3.1 GH treatment

Female transgenic mice, dwarf rats and their wild-type controls were set up for mating with males between 4-6wks of age. Date of assumed pregnancy was determined by daily plug checks. Pregnant mice and rats at day 16 were given a single intravenous injection of varying doses of recombinant bovine growth hormone (bGH) or saline through the tail vein. Animals were culled 25 minutes following injection and blood and tissues were collected as appropriate. Six animals were used for each group.

2.3.2 Continuous GH treatment

Pregnant day 12 animals had osmotic minipumps (Alzet) inserted subcutaneously under isoflurane anaesthesia. Analgesic drugs were given immediately post-operatively. Pumps delivered a daily dose of 200µg of bGH/bodyweight. After 7 days the animals were culled, and tissue and blood samples collected.

2.3.3 Fasting experiments

Pregnant day 16 animals were fasted for 48 hours. On the 48th hour the fasted animals were given a single intravenous injection of bGH through the tail vein. Another group of fasted animals were given an additional single subcutaneous injection of 10mU/g of insulin (Humulin) at the 40th hour into the fast was followed by a single injection of bGH at the 48th hour of the fast through the tail vein. All animals were culled 25 minutes after the final injection.

2.4 Immunocytochemistry of paraffin embedded sections.

Dissected tissues were stored in 4% PFA in PBS for 15 hours at 4^{o}C before being transferred to 70% ethanol. All tissues were embedded in paraffin and sectioned (6µm) and stored at 4^{o}C until use. I am grateful to Wendy Hatton (histology services, NIMR) who performed this. Tissue sections were dewaxed using histoclear (National Diagnostics) for 5 minutes to remove paraffin, followed by rehydration in descending concentrations of ethanol (2 minutes each in 100%, 70%, 30%, dH_2O). To minimize background due to endogenous peroxidase activity, sections were incubated in 1% H_2O_2 diluted in 40% methanol. To enhance antigen retrieval, sections were incubated in 0.05% pepsin (Sigma) in 10mM HCl at 37^{o}C for 7 mins or 0.05% trypsin in 0.1% $CaCl_2$ (Sigma) for 15 minutes. Non-specific binding was blocked by incubating

sections for 1 hour at room temperature in TNB blocking solution (0.1M Tris-HCl (pH 7.5), 0.15M NaCl, 0.5% Blocking Reagent (PerkinElmer, 0.5% Triton x100). The primary antibody was diluted in blocking solution and incubated with sections at 37^{o}C overnight. After overnight incubation, unbound primary antibody was removed by washing with TNT (0.1M Tris-HCl, 0.15M HCl, 0.05% Tween-20) 3x for 5 minutes. Sections were then incubated with secondary antibody at 37^{o}C for 45 minutes. Sections were washed twice for 5 minutes in TNT and once in TN (lacking tween) to terminate antibody reaction. This was then followed by an Avidin-biotin step (ABC Elite Kit, Vectorstain) for which the sections were incubated for 30 minutes at room temperature. Some sections were then treated with biotinylated tyramides (PerkinElmer) for 15 minutes at room temperature in the dark to amplify the staining reaction. Sections were then washed 3x for 5 minutes with PBS before being incubated for 1 minute in diaminobenzidine (Sigma) to visualize peroxidase labelled antibodies, and then counterstained in Methyl Green (pH 4.5).

2.5 Hormone analysis

2.5.1 Tissue collection and preparation

Pituitaries were then dissected and homogenised on ice in 1mL cold PBS. Homogenates were stored at -20^{o}C until assayed.

2.5.2 Radioimmunoassay (RIA)

RIA Solutions

PBS	**50mM NaH_2PO_4, 100mM NaCl, 0.6mM Thimerosal, pH 7.4**
Tris Buffer	**100mM Tris-HCl (pH 8.4), 0.6mM Thimerosal.**
18% PEG	**2mL 10%Trition x100, 1.5g γ-globulins, 330mL Tris buffer, 667ml 27% polyethylene glycol (PEG), water to 1L**
RIA Buffer	**PBS-0.3% BSA.**

2.5.3 Hormone Iodination

Hormone iodination was performed using the iodogen method adopted by Danielle Carmignac in our lab, this was followed by the separation and elution of iodinated hormone from unbound iodine-125, carried out on 0.6 x 26cm Sephadex G75 columns (Amersham Bioscience). Prior to use, the columns were equilibrated with PBS/BSA (0.3% w/v BSA). Hormones for iodination were stored at -20^{o}C in 0.2M sodium phosphate solution (pH 7.4) as 10µg aliquots. Immediately prior to iodination, a further 10µg of 0.2M sodium phosphate solution was added to the hormone solution. Iodine-125 (^{125}I, Amersham) was stored at 4^{o}C in 20MBq aliquots. An aliquot was diluted by the addition of 15µL of 0.2M sodium phosphate immediately prior to use. Hormone was added to the diluted ^{125}I, and the mixture transferred to a glass tube containing 5µg of dried iodogen (1,3,4,6-teracholride-3α,6α-diphenylaglycouril; Sigma), and incubated at room temperature for 5 minutes. A total of 220µL of 0.2M sodium phosphate solution was then added and incubated for a further 2 minutes. This mixture was then added to the top of the column and eluted using PBS containing 0.3% w/v BSA. Fractions (1mL) were collected manually and the radioactivity

counted. The middle fraction with the highest MBq count containing the labelled hormone was diluted into aliquots and stored at -20^{o}C.

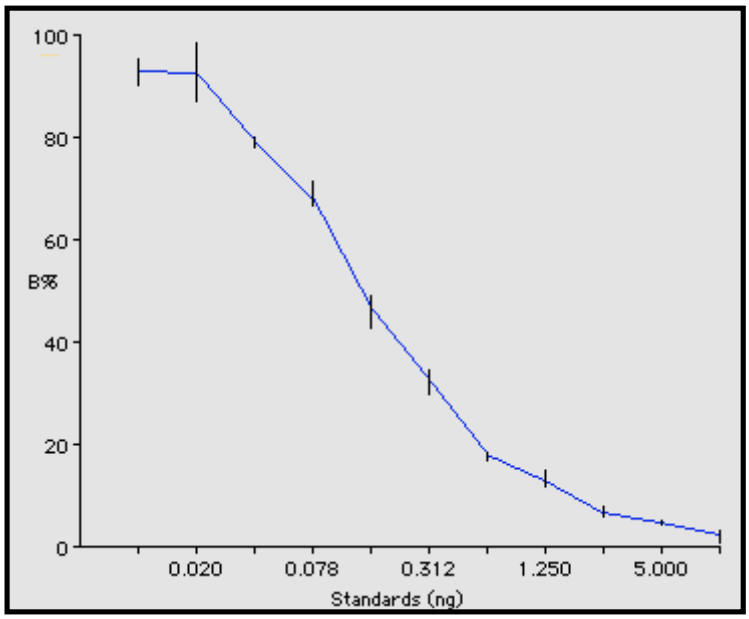
2.5.4 Standard Curve

The standard curve was prepared in triplicates in PBS/BSA. Total counts (T), antibody blank (ABL) and non-specific binding (BO) were used as controls were as follows:

- T- 100µL iodinated hormone (diluted to give 5000-10,000cpm) only.
- ABL- 200µL PBS/BSA + 100µL iodinated hormone.
- B_o- 100µL PBS/BSA + 100µL antibody +100µL iodinated hormone.

Standards were set up in triplicates as two-fold serial dilutions from a top standard of 5ng hormone in PBS/BSA in a volume of 100µL per tube. Samples were set up as duplicates, also in two-fold serial dilutions. An antibody solution (100µL) and 100µL of iodinated hormone were then added to each tube. Antibody concentrations and standards are shown in Table 2.3. Tubes were shaken and incubated at room temperature for 24 hours. After incubation the antibody-bound hormone fraction was precipitated using 600µL 18% PEG-IGG solution. Tubes were allowed to stand at 4^{o}C for 30 minutes, before being separated by centrifugation at 3000g for 12 minutes at 4^{o}C. The supernatant was then aspirated and the tubes capped and placed in a gamma counter to measure the radioactive ^{125}I in the pellets. Counts were averaged over 3 minutes. The percentage B_o was then calculated for each triplicate averaged and compared to the standard curve. A typical standard curve for mouse IGF-1 is shown in Fig 2.1, a best line of fit shown for the triplicate values obtained for each standard.

Figure (2.1) Typical mouse IGF-1 RIA standard curve.



50% Bo=0.39698ng, ABL=5.19%, Bo-26.06%

Each standard was run in triplicate, and values between Bo 30-80% on the standard curve were read. The readings would then be multiplied according to dilution factor to generate ng/mL serum as plasma.

2.5.5 Pituitary samples: GH and PRL

Standards and samples were always run together in each assay. Two-fold serial dilutions of the samples were set up in duplicate in PBS/BSA with 100µL final volume. Antibody solution and iodinated hormone were added and incubated at room temperature for 24 hours. After incubation the bound hormone fraction was precipitated using 18% PEG solution. The percent B_o was then calculated for the average of each set of duplicates (using a RIA plot program), to determine the mean ng of hormone per duplicate. This was then converted to µg per pituitary. 50% B_o values for GH, PRL and IGF-1 RIAs are shown in Table 2.2, indicating the mid point

on the respective standard curves. All the samples were rapidly measurable in these assays.

Table (2.2) 50% BO values for GH, PRL and IGF-1 RIA for mouse and rat.

RADIOIMMUNOASSAY	MOUSE	RAT
GH	0.126ng	0.158ng
PRL	0.431ng	0.290ng
IGF-1	0.397ng	0.116ng

GH and PRL measured as pituitary content. IGF-1 measured from plasma.

2.5.6 IGF-1 RIA

In order to separate IGF-1 from its binding proteins prior to assay, plasma samples were extracted on the day of assay using a previously published protocol (Ebensperger *et al* 1998, [287]). Briefly, the samples were diluted 1:4 in acid-alcohol (87.5% ethanol, 12.5% 2M HCl), vortexed, and incubated at room temperature for 30 minutes. These were then separated by centrifugation at 0.8g for 10 minutes at 4^{o}C. The supernatant was removed and mixed in a 5:2 ratio with 0.855M Tris base to neutralise the solution. This was vortexed and incubated at -20^{o}C for 1 hour. Following the incubation, samples were separated by centrifugation at 0.8g for 30 minutes at 4^{o}C. The supernatant (20µL) was then used for the assay and made up to 100µL assay volume with PBS/BSA. Tubes were incubated and processed as for the pituitary extracts. Results were converted to ng/mL.

Table (2.3) Antibodies and standards used for RIA

ASSAY	ANTIBODY	FINAL ANTIBODY CONCENTRATION	STANDARDS	STANDARD RANGE
mGH	Monkey alpha-rat GH NIDDK	1:1,500,000	mGH NIDDK	10ng-10pg
mPRL	Rabbit alpha-mouse PRL NIDDK	1:400,000	mPRL NIDDK	5mg-20pg
mIGF1 & rIGF1	DSL	1:168,000	mIGF1 & rIGF1 DSL	10ng-10pg
RGH	Monkey alpha-rat GH NIDDK	1:300,000	rGH NIDDK	10ng-10pg
RPRL	Rabbit alpha-mouse NIDDK	1:400,000	rPRL NIDDK	10ng-10pg

2.6 DC Protein Assay (detergent compatible)

The detergent free protein assay (Bio-Rad) *DC* Protein was used to measure protein concentration following detergent solubilization. The reaction is similar to the well-documented Lowry assay [288], but is faster and the color is more stable. A standard curve consisted of 3 - 5 dilutions of a top protein standard containing 1.5mg/mL to about 0.2 mg/mL of protein. Each of these standards as well as samples (5 μL) was pipetted into a microtiter plate followed by addition of manufacturers reagents. The plate was mixed for 5 minutes on a plate mixer and the absorbance was read at 750 nm.

2.7 Western Blot protocol

Liver and placental primary tissue was homogenised in with RIPA buffer (50mM Tris-HCL (pH 7.6), 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail tablet (1x 25U tablet/10mL, Roche), and stored at -80 ^{o}C until further use. Loading buffer (100uL; 50mM Tris-HCL (pH 6.8), 100mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) was added to each sample. Samples were denatured at 95^{o}C for 5 minutes, then loaded onto a miniture 10% polyacrylamide gel (Biorad) and separated by electrophoresis at 150V in a buffer solution (25mM Tris Base, 250mM glycine, 0.1% SDS; in dH_2O). Samples were transferred to Nitrocellulose membrane (Hybond) for 2 hours in 1x transfer buffer (30mM glycine, 48mM Tris Base, 0.0037% SDS, 20% methanol; in dH_2O), which was then incubated with primary antibody overnight at 4^{o}C. Non-specific binding of the antibody to membranes was blocked by incubation in blocking buffer (5% milk powder in TBS-T (0.1% Tween20 in TBS). After washing in TBS-T, the membrane was incubated with mouse HRP-conjugated secondary antibody (1:10,000) in blocking buffer for 1 hour at room temperature. Specific antibody binding to membrane was detected by incubation with Supersignal® Chemiluminescent Substrate (Pierce) for 5 minutes before exposing to film.

2.8 Microarray

2.8.1 RNA Isolation

Total RNA from rat placenta was isolated using a TRIzol reagent protocol (Life Technologies). Tissue samples were homogenized in 1mL of TRIzol reagent per 50-100mg of tissue and incubated for 5 minutes at 15-30^{o}C to ensure the dissociation of nucleoprotein complexes. This was followed with the addition of 0.2mL of chloroform

per 1mL of TRIzol reagent, and incubation at 15-30^{o}C for 3 minutes. The aqueous phase formed was removed and transferred to fresh tubes with 5mL of isopropanol per 1mL of TRIzol reagent originally used. Samples were then incubated at 15-30^{o}C followed by centrifugation at 2-8^{o}C for 10 minutes. The supernatant was then removed and the remaining RNA precipitate was washed with 75% ethanol, 1mL of ethanol per 1mL of TRIzol used. Samples were centrifuged at 7500x g for 5mins at 8^{o}C, and the RNA pellet was then air dried for 5-10 minutes.

2.8.2 Microarray experimental protocols

A quantity of 500ng of total RNA was subjected to the Affymetrix small sample two-cycle protocol (Affymetrix GeneChip® Two-Cycle cDNA Synthesis Kit, Affymetrix Inc, Santa Clara, CA, USA). This protocol is designed to prepare labelled cRNA from as little as 100ng of total RNA. The resultant biotin labelled cRNA was then used to hybridize to GeneChip® arrays. Before hybridization the labelled cRNA product was assessed in a 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA), followed by fragmentation of 12μg of the labeled cRNA products following Affymetrix protocols. Each sample from the different experimental conditions was then processed and run on a separate rat gene chip. The fragmented cRNA products were firstly hybridized to GeneChip Test3 gene arrays to assess cRNA quality, and then to rat 230 2.0 array (900505) GeneChip array (Affymetrix), for 16 hours at 45^{o} C following standard Affymetrix protocol. Following the 16-hour hybridization, bound biotinylated cRNA was then targeted by fluorophore conjugated-streptavidin. The signal amplified by the use of biotinylated anti-streptavidin antibody.

Following washing and staining, the arrays were then scanned on the Affymetrix 3000 7G scanner. Data files from generated gene transcript levels were then determined using the PLIER algorithm and Genespring (version 9) by Abdul Sesey.

2.9 In vitro Analysis of human placental choriocarcinoma cell lines

2.9.1 Tissue culture

Human BeWo and Jar1 placenta choriocarcinoma cell lines were obtained from American Type Culture Collection (ATCC). Cells were maintained as a monolayer in modified culture medium recommended and obtained from ATCC. For the BeWo cell line, F-12K (Kaighn's modification of Ham's F-12 Medium) was used. RPMI-1640 medium was used for the Jar1 cell line. Hep2 cells were maintained in DMEM medium. All medium was supplemented with 10% fetal calf serum and 50mg/mL gentamycin (Invitrogen).

2.9.2 Cell line transfections

(i) Nucleofection

Cell lines were cultured as normal and passaged two days prior to nuclear transfection using electroporation. Cells were transfected at 70% confluency using nuclear transfection kits (Amaxa). Table 2.4 shows a summary of the optimal conditions used for the given cell lines using nuclear transfection.

Table (2.4) Conditions used to transfect Jar1, BeWo and Hep2 cells

CELL LINE	AMAXA ELECTROPORATION PROGRAMME	BUFFER	DNA TRANSFECTED	AMOUNT OF DNA (μG)
Jar1	A20	L	GHR/GFP/Stat5bGFP	1
BeWo	A20	V	GHR/GFP/Stat5bGFP	1
Hep2	I-13	R	GHR/GFP/Stat5bGFP	1

Transfected cells were plated on polylysine-coated coverslips (Sigma) and maintained in growth medium at 37^{o}C and 5 % CO_2 for the period of the experiment, usually 48hrs.

(ii) lipofection

Cells were plated in 24-well plates on polylysine-coated coverslips. After 24 hours the cells were transfected using Lipofectamine 2000 (Invitrogen) as described in manufacturer's reagent protocol. Briefly, 1μg of DNA was diluted in 50μL of serum free medium and mixed gently. Lipofectamine 200 (2μL) was diluted in 50μL of serum free medium, mixed and incubated at room temperature. After 5 minutes, the diluted DNA and lipofectamine 200 were mixed together and incubated at room temperature for 20 minutes. The DNA–Lipofectamine mix was then added to each well containing cells and medium (500μL serum free), and the plates rocked gently at 37^{o}C for 5 hours. The transfected medium was then replaced with growth medium and returned to the 37^{o}C incubator for the remainder of the experiment.

2.10 Immunofluorescence

Cells on coverslips were removed from growth medium and placed in 4% PFA (Sigma) and fixed for 15 minutes. This reaction was quenched and the cells permeablized using 50mM NH_4Cl and 0.02% (w/v) saponin (Sigma) for 15 minutes. Cells were then placed in PGAS (0.2% gelatin (Sigma), 0.02% saponin) for 5 minutes

before incubating with the primary antibody (in PGAS) for 45 minutes. Primary antibody was removed by washing with PGAS and then the cells were incubated with secondary antibody for 30 minutes. Cells were washed in PBS followed by dH_2O and mounted using Mowiol (100% glycerol [Sigma], 40% [w/v] Mowiol 4-88 [Calbiochem] in ddH_2O), ± DAPI (4', 6-Diamidino-2-phenyindole, Dilactat). All incubations were carried out in the dark at room temperature.

2.11 Imaging

2.11.1 Image capture and analysis

Light microscope images were captured on an AxioPlan2 microscope using Openlab software, version 5. Fluorescent images were captured using a Leica TCS SP. All images were captured both at high magnification (x 40) and low magnification (x 10). All light microscope images were quantified using a module of the Java-based image-processing programme, ImageJ (Wayne Rasband).

2.12 Statistical analysis

Unless otherwise stated, all results were presented as mean ± S.E.M and statistical analysis was carried out using Instat 2.01. Where two groups were compared, statistical analysis was performed with unpaired Student t-test. Where multiple groups were compared, analysis of variance (ANOVA) followed by post-testing of selected pairs with Dunnetts, Bonferroni, or Newman Keuls tests was used to determine the groups showing significant differences. Significance values are shown as: * $P<0.05$, **$P<0.01$, *** $P<0.001$ in this thesis.

Results

3. Models of GH signaling in the placenta *in vitro* and *in vivo*

3.1 Human choriocarinoma cell lines and their use in studying GH signaling

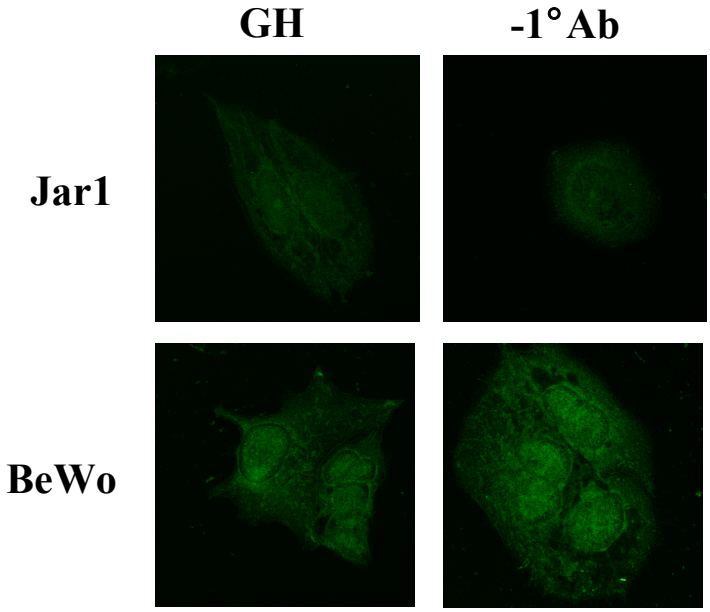
Cell lines considered to be representative of the human trophoblastic lineage were readily available at the outset of my studies. Whilst waiting for the transgenic animals from the Mendelson lab, I thought I would see if I could establish a GH-signaling system in human placental cell lines. How representative these different types of cell lines really are of the human placenta is a point of debate and discussed in chapter 1. Direct effects of GH are notoriously difficult to obtain in *vitro* (hence the somatomedin hypothesis) but I felt it important to utilize the availability of such tools, to see if I could use *in vitro* to models to establish responses to GH, for later application *in vivo*.

I used two different choriocarcinoma cell lines, BeWo and Jar1 [289, 290] derived from spontaneous choriocarcinomas. These cell lines have been available for over 30 years with a large literature. Both cell lines are reported to be representative of the human trophoblast lineage owing to their reported production of placental hormones, including reports of the growth hormone variant [283].

Owing to the similarity between the protein sequence of GH and GH-V, an existing in house radioimmunoassay (RIA) using a polyclonal sheep hGH antibody was used to measure GH in the two cell lines. This RIA detected very small amounts of GH for the BeWo cell line, but no measurable amounts of GH for the Jar1 cell line, (data obtained was < 40pg in 100μL of sample). One explanation for the detection of such low levels of GH in the cell lines may have been due to the lack of sensitivity of the RIA, I therefore attempted to make the assay more sensitive by the late addition of tracer, however, no measurable difference in GH was detected in either cell line.

Furthermore, I also tried a ELISA assay kit specific for GH-V. Results from this assay also showed no measurable amounts of GH-V for either cell line. The ELISA assay was designed to typically measure GH-V levels in blood samples, which may have accumulated higher levels than produced *in vitro* from cells. Nevertheless, with the assays I had, neither of the cell lines was going to be useful for measuring regulation of the production of GH-V, I thought this could mean they might be useful to study the effects of endogenous GH. I therefore carried out a number of immunofluorescence experiments on both cell lines to visualize the expression of GH receptors. Interestingly, faint immunofluorescent staining was present, though variable in the BeWo cells, but undetectable in the Jar1 cells (Figure 3.1).

Figure (3.1) Immunofluorescent staining for GHR in Jar1 and BeWo cells



3.2 Transfection studies in placental cell lines

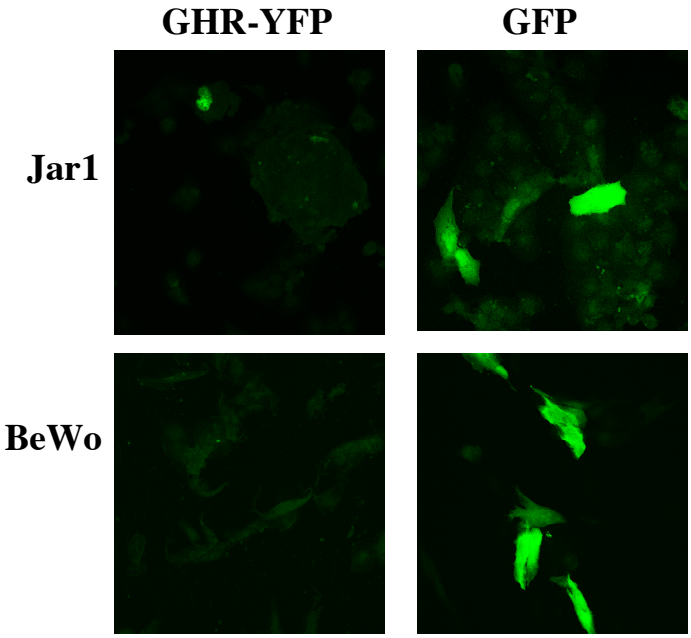
Given the faint staining in these cultured placental cell lines I wished to try to improve detection and use these as a basis for introducing components of the GH signaling cascade i.e. the GHR into the cell lines, by transfection. To clarify whether a transfected GHR was functional, cells were exposed to GH, and then I attempted to detect a GH-GHR interaction by visualization of phosphorylated Stat5, as has been successfully achieved in chondrocytes and liver [291]. I used lipofection to introduce GHR to the placental cell lines, with a GHR construct (GHR-YFP) with a fluorescent tag in order to assess transfection efficiency, as previously used by Gevers here in the lab (2009) [291]. Although, this lipofection method has been previously used to transfect BeWo cells and been successful [292] I could not rescue viable placental cells following lipofection with this construct.

I therefore switched to nuclear transfection, using a kit from Amaxa, which uses nuclear electroporation to introduce constructs. To my knowledge, this method, quite new at the time, had not been previously used to transfect placental cell lines, but several members of our lab had used this method to transfect a variety of other cell types, including endothelial cells, achieving high levels of viable transfected cells. I therefore tested several of the buffers and protocols supplied by the manufacturers, transfecting both GHR and GFP (as a positive control) for the individual placental cell lines. This was somewhat more successful than lipofection, and viable cells following transfections could be identified under a fluorescence microscope, and the transfection efficiency calculated by counting fluorescent cells from five randomly selected fields.

However, few placental cells remained viable following nuclear transfection with GHR under most conditions, so the combination of the highest transfection efficiency, with the lowest cell death, in reference to both GFP and GHR transfected cells was sought. These conditions were program A20, with buffers V and L, for BeWo and Jar1 cell, respectively. The details about the buffers and programs are kept confidential by the manufacturers, so it is difficult to suggest why these specific conditions were more optimal for transfection efficiency.

The conditions outlined above were then used to transfect cells with a range of construct concentrations, to determine an optimal amount of DNA for transfection, measuring randomly selected fields over a time course of 6-48 hours. I found the optimal amounts of GHR DNA to transfect were 1μg and 6μg, for BeWo and Jar1 cell lines, respectively, with an incubation time of 24 hours. Figure 3.2 illustrates cells from both placental cell lines transfected with GHR and GFP under optimal conditions. GFP was bright; GHR-YFP was dimmer but a few YFP positive cells were clearly visible under the microscope. Both Jar1 and BeWo cells illustrated in Fig.3.2 were transfectable but stronger signal was seen for GFP, compared to the GHR construct.

Figure (3.2) Jar1 and BeWo cell transfected with GHR-YFP and GFP

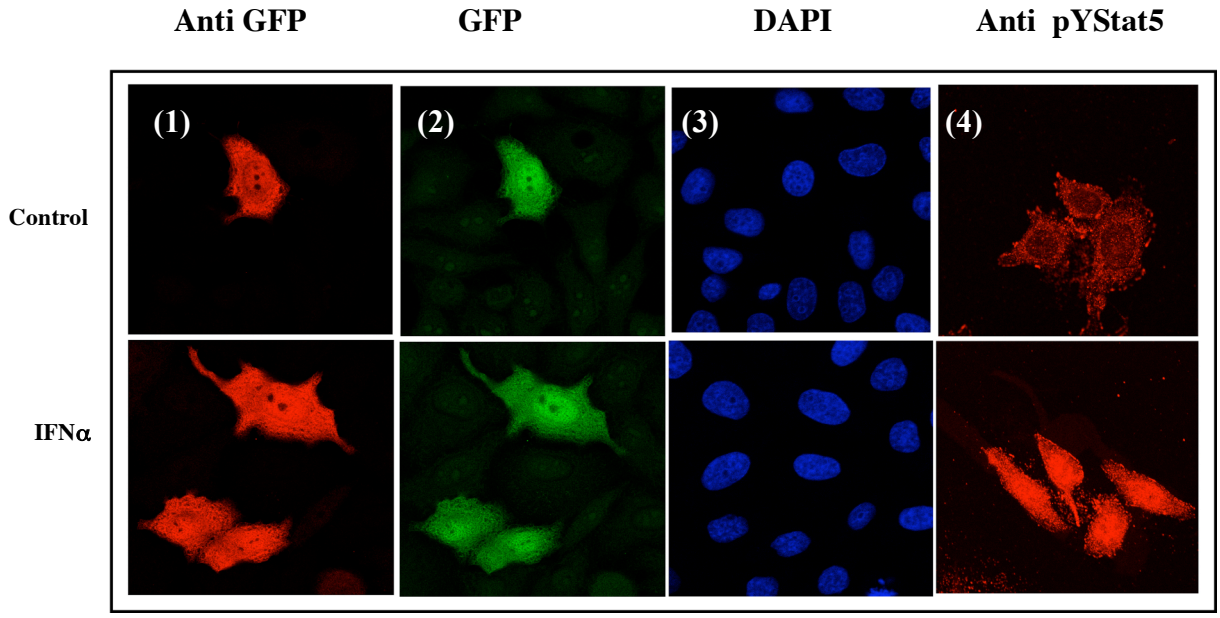


Since the fluorescence signal from GHR-YFP was faint, I used immunofluorescent staining in an attempt to improve the visualization of the few GHR transfected cells more clearly, however, the level of signal observed for the GHR transfected cells remained low, even with confocal microscopy. Nevertheless, I incubated some of these cells with GH to try to stimulate Stat5 phosphorylation, in case this would give amplification. However, the level of GHR was either too low, or in too few cells, to see a significant Stat5b response. One reason for the poor success of GH signaling could be that many components of several signaling pathways may be compromised in the placental cell lines due to their pathological origin. For example low endogenous expression levels of GHR could also be accompanied with low levels of Stat5 after many passages. I therefore attempted to carry out double transfections with GHR and a Stat5b tagged construct (Stat5bGFP, a kind gift provided by Carter-Su *et*

al (1999) [293]. The placental cell lines were transfected with Stat5bGFP or double transfected with GHR-YFP and Stat5bGFP, cultured in GH, and then stained for phosphoStat5. This time I included control cells of non-placental origin to check whether the lack of signal was due to my poor technique or bad reagents causing these negative results. Hep2 cells, derived from squamous cell carcinomas were used. These cells express interferon alpha-receptors, like GH, a member of the cytokine nuclear receptor family. Upon ligand (interferon alpha) binding to its receptor, members of the Stat family are shown to be phosphorylated and translocated to the nucleus in these cells [E.Gevers in our lab, personal communication]. I therefore felt that cells known to be capable of initiating the phosphorylation of Stat5, without the need of an exogenous supply of Stat5 would be a good positive control. With the successful transfection of the Stat5bGFP construct in these cells, I would also expect to be able to track the translocation of phosphorylated Stat5bGFP upon IFNα stimulation.

This would provide good evidence that the transfected Stat5b construct was capable of being phosphorylated, the stimulation worked and would indicate the viability of cells following transfections using this construct. Figure 3.3, illustrates the results obtained for the Hep2 control cells, Figure 3.4 (a) and (b) illustrates immunofluorescent staining for pYStat5 for both Jar1 and BeWo cell lines, respectively.

Figure (3.3) Hep2 cell lines transfected with Stat5bGFP, +/- IFNα treatment

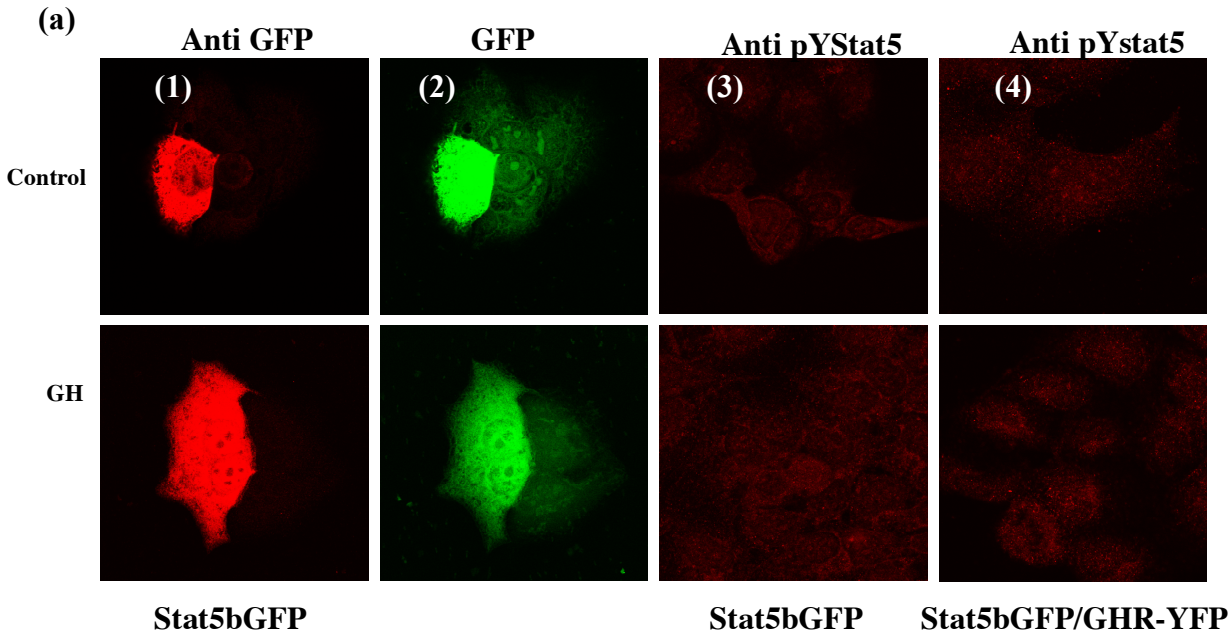


The top panel of images show Hep2 cell transfected with Stat5bGFP; image (1) shows fluorescent staining for GFP part of the Stat5bGFP (2) shows the fluorescence observed for the GFP without immunofluorescent staining (3) nuclei stained by DAPI, (4) Immunofluorescent staining for Hep2 cells following no treatment with IFNα. The lower panel shows the same groups, treated with IFNα. Images were created on a confocal microscope, x100

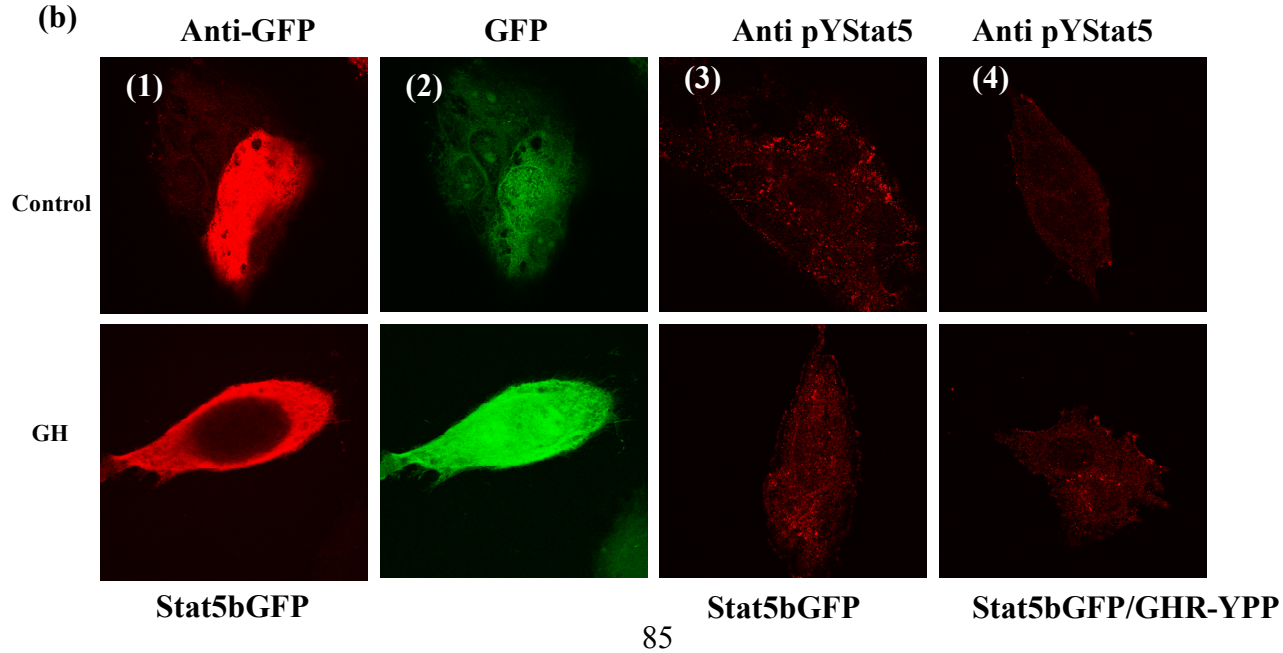
The confocal images shown in figure 3.3 clearly indicate that Hep2 cells are transfectable with the Stat5bGFP construct. Furthermore, the Dapi staining of the cells confirm the survival of cells during the transfections. Transfected Hep2 cells can be clearly identified by the fluorescence shown by the GFP part of the construct alone, as well as by immunofluorescent staining for GFP. Most importantly, administration of IFNα clearly shows the translocation of the fluroscence to the nuclei of cells, when compared to controls, thus verifying the phosphorylation of the Stat5b construct upon IFNα stimulation. This was encouraging as it suggested that my technique and reagents were functioning in these control cells, but suggested the

placental cell types were less good models for this *in vitro* approach, as my technique and reagents were the same.

Figure 3.4 (a) Jar1 (b) BeWo cells transfected with GHR or GHR/Stat5bGFP



(a) Top panel shows images for Jar1 cells; image (1) Stat5bGFP transfected Jar1 cell immunostained for the GFP part of construct, (2) fluorescence from a Jar1 cell for the GFP part of the construct, (3) Sta5bGFP transfected Jar1 cells immunostained for pYStat5, (4) immunofluorescent staining for double transfected (Stat5bGFP/GHR-YFP) Jar1 cells. Lower panel shows the same images but for cells treated with GH. Fig.3.4 (b) Shows images for the same conditions but for experiments carried out in BeWo cells.



Confocal images shown in both Figure 3.4 (a) and (b) show that the Jar1 and BeWo placental cell lines could be transfected with the construct Stat5bGFP, although the number of cells were much fewer than Hep2 cells. As with the Hep2 cells the GFP part of the construct could be visualized with use of fluorescent microscopy as well as with fluorescent immunostaining for GFP. Unlike the Hep2 cells both Jar1 and BeWo cells showed no translocation of the construct upon GH stimulation. This was a disappointment as it suggested that any undetected endogenous GHR as well as the few GHR transfected cells remain unresponsive to GH even through the supply of exogenous signaling components. Given the primary aim of my thesis was to pursue a GH response *in vivo*, I didn't embark on any further attempts to set up a cell-line system based on these cells, and concentrated on rodent placental responses *in situ*. As will become apparent in chapter 4, this proved a more fruitful approach. However, it is important to note that other placental cell lines might be better suited to this approach.

3.3 Discussion

It seemed reasonable to look first at placental cell lines to see if they could reflect the human placenta, at least in terms of investigating the effects of GH and GHR signaling. Studies by Frankeene [229] show GH production and GHR expression in the syncytiotrophoblast cells of the human placenta, and BeWo and Jar1 cells have been documented for being representative of the syncytial population [283] so there was reason to believe that these cells might also show GH production and endogenous GHR expression. My attempts to measure GH production from the placental cells lines proved difficult, with both methods employed being able to only show small amounts of GH production from one cell line. It is probable that assay sensitivity may

not have been sensitive enough to detect very low levels of GH production, and with hindsight, I could have established this with more sensitive methods like *in situ* hybridization. However this could not have given me the experimental system I needed, and paradoxically, the lack of endogenous GH could be a benefit for studying signaling in response to exogenous GH.

Following immunofluorescence staining for GHR, very little flurorescent staining was observed in BeWo cells, (and not very convincingly specific) compared to antibody controls. As with GH production, no GHR staining was observed in the Jar1 cells, further emphasizing the variation in the properties of the two placental cell line.

With the tumourous origin of the cell lines in mind, it is highly likely that the morphological and functional state of the cell lines may be altered when compared to that of normal human placenta. But in any event, the cell lines did not serve my purpose. Even when transfected with both GHR and Stat5b constructs, these cell line did not mount good Stat5 responses to GH; even after extensive optimization trials, very few cells were successfully transfected with GHR-YFP or Stat5bGFP. The number of transfected cells wasn't overly convincing, and for those with signal, the Stat5 response to GH was rather inconclusive, especially when compared to the robust IFNα responses in the control Hep2 cells.

In conclusion, despite the exogenous addition of two major components of the GH signaling cascade, I could not reconstitute responses to GH in these cell lines.

The placental cell lines could be lacking in a number of other components required for GH signaling cascade, but the combination of endogenous absence of GHR, the low transfection efficiency (eg. JAK kinase), and the inability of the cells to show function

through transfected GHRs and Stat5s made me decide to not pursue this route further in *in vitro* models for the study of GHR activated signaling. It should be remembered that many studies in many different tissues have also found it difficult to obtain reliable robust GHR signaling, hence the somatomedin hypothesis, though there are now some successful GH-responsive cell lines (Gevers *et al* 2009) used in the lab.

Having spent a lot of time on this, and with no guarantee that the placental cell lines would generate any results, I felt it more constructive to move on to a different model to observe the possible effects of GH on the placenta, I therefore decided to utilize the GH deficient rodent models available in my division. Whilst this has the advantage of an *in vivo* approach, it does loose the advantage of human system that the cell lines offered. This was a feature I needed to bear in mind in the following rodent studies presented in this thesis.

3.4 GH-deficient rodent models

3.5 Introduction

Even though GH is produced in the fetus from around the third month of gestation in humans [294] and from embryonic day 16-19 in rodents [295, 296] most available evidence suggests that pituitary GH does not have a direct role in fetal growth and development. For example fetal sheep made GH deficient by hypophysectomy are of normal birth weight [297]. Similarly, human infants with congenital GH deficiency show only a small reduction in birth length [298]. This has also been confirmed in newer studies with Laron Dwarfs, which show normal birth weights despite having defective GH receptors [299].

Despite this, there are high levels of circulating GH in the later terms of both human and rodent pregnancy. The role of this remains unclear, however, a potential indirect role could be considered, via effects on other processes like metabolism, not as obviously reflected in skeletal growth as it is in post-natal life. In the experiments described in this part of chapter 3, my specific aims were to (i) briefly describe the characteristics of GHD rodent models used, and measure their pituitary hormones in pregnant vs non-pregnant states, which has not previously been reported and (ii) to observe whether there is a change in some pituitary hormones during pregnancy in these models. All measurements were taken at approximately day 16 of both rat and mouse pregnancy, with all animals being set up for mating after 4-6 weeks of age. I also tested whether GH-deficiency during pregnancy in these models would affect such outcomes as the weight of mother, pups, and number

of pups born to each mother. Differences if any in these parameters between GH-deficient and normal mothers would of course also reflect possible consequences of a small mother on fetal growth, which might imply a possible indirect role of GH status in pregnancy, as well as other factors relating to uterine size, not directly depending on GH.

3.6 Hormone analyses in pregnant Dwarf and AS rats

Our lab was the first to identify the dwarf rat model used in my experiments. These dwarf rats *(dw/dw)* have an inherited autosomal recessive mutation (as yet uncharacterized) resulting in a severe but sub-total GH deficiency [300, 301]. Chromosomal location studies (Le Tissier, unpublished) have excluded all the known candidates genes affecting the GH axis but the locus close to a centomere has proven difficult to clone. The initial characterization of the dwarf (*dw/dw*) strain showed that post weaning growth was retarded, 3 month old male and female rats showed weights approximately 40% less then their normal litter mates, and continued to grow at a slower rate. Pituitary GH concentrations were approximately 10% of normal in male and 6% in female rats, all other anterior trophic hormones (LH, TSH, prolactin and ACTH) were within the normal range [300]. Since these initial observations by the Charlton group, several other studies have measured the same hormones, as well as, carried out more detailed work to characterize the *dw/dw* model further [301, 302]. Studies in *dw/dw* rats have shown their acute response to GH secretagogues such as GH-releasing hormone (GHRH) or growth hormone releasing peptide-6 (GHRP-6) as being reduced in proportion to their pituitary content [300, 303]. One defect appears to be an inability to generate a cyclic AMP response to the administered GHRH, which results in a failure to build pituitary stores. A compensatory effect of their

somatotroph hypoplasia appears to be an increase in prolactin (PRL) and PRL cell number [304, 305]. Subsequent measurements have shown significantly higher pituitary PRL content in adult *dw/dw* animals, although their basal plasma PRL levels were normal [305, 306]. The pituitary content for GH and PRL has never been measured in pregnant dwarf rats, so as well as providing new data, my proposed studies would address the question of whether pregnancy changes the magnitude of GH deficiency or prolactin excess observed in non-pregnant dwarfs, compared to pregnant normal Albino Swiss (AS) rats. I also measured IGF-1 plasma levels in the same groups of animals, as a more general index of GH activity.

Table 3.1 shows pituitary GH contents from pregnant and non- pregnant dwarf and (AS) rats. As expected, the pituitary GH content in non-pregnant rats was profoundly reduced in dwarfs (3.2±0.7), compared to normal AS rats (181.2±23.8), $P<0.001$. My new findings were a similar significant reduction in the pituitary GH content in pregnant dwarfs (3.0±1.7) vs AS rats (186±34.4), $P<0.001$. However, I noted that when comparing the pituitary GH content of non-pregnant with pregnant rats, no significant differences were found within either strain. Since fasting in rodents induces a state of GH resistance and reduced GH levels [80], and I planned to use fasting in later experiments, it was also of interest to measure the pituitary GH contents of fasted pregnant rats. Measurements of pituitary GH contents in an additional group of pregnant 48 hour fasted, normal and dwarf rats (Table 3.1) showed no significant differences in pituitary GH, when compared to the non-fasted pregnant groups. Importantly, fasting did not build up GH stores in dwarf rats.

Table (3.1) Pituitary GH content measured for normal (AS) and dwarf (pregnant and non-pregnant) rats.

RAT MODEL	NON-PREGNANT	PREGNANT	PREGNANT/FASTED
AS	181.2±23.8	186±34.4	186±29
Dwarf	3.2±0.7***	3.0±17***	3.8±1.7

Pituitary contents shown for (6wk +) old animals at day 16 of pregnancy

An increase in the baseline circulating GH concentrations is evident in pregnant rodents [81] and recent data suggest that the increase in GH levels during pregnancy is likely to be a result of increased secretory activity [307]. Since I found no change in GH contents between pregnant and non-pregnant rats, this could imply that the production of pituitary GH would also be increased during pregnancy, to match increased secretory activity. Dwarf rats show no intrinsic impairment in responding to GRF treatment with GH secretion, however, the amount of GH secreted is much lower than normal rats, reflective of their reduced pituitary GH content [303]. I thus feel is reasonable to assume that any increase in GH secretion during pregnancy in the dwarf rat will not make a significant contribution to plasma GH, and it certainly does not restore the GHD state to normal.

3.6.1 Pituitary prolactin content in non-pregnant and pregnant dwarf and AS rats

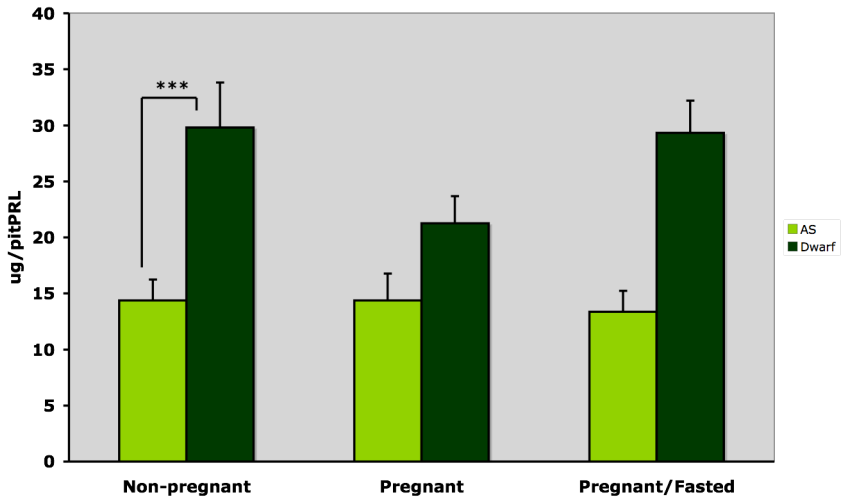
During pregnancy and in preparation for lactation there is a progressive increase in hypophysial PRL secretion, with an extensive modification of lactotrophs, increase in the synthesis and secretion of prolactin (PRL) [308]. As mentioned above, dwarf *dw/dw* rats are unusual in having relatively high PRL contents despite having low GH [309] but again, PRL measurements had not been made in pregnant *dw/dw* rats, so I

wanted to measure the PRL pituitary contents during pregnancy in AS and *dw/dw* rats (Fig 3.5). I found a significantly higher level of pituitary PRL content in non-pregnant dwarfs (29.81±4.0), compared to non-pregnant AS rats (14.3±1.9), $P<0.001$. The original findings from Charlton *et al* (1988) [300], in which pituitary PRL content measured in small groups of dwarf rats, found no differences, but the same group later analyzed larger groups of animals on the same genetic background, and found larger differences. My results support these later findings, confirming that *dw/dw* dwarf rat have increased pituitary PRL contents, (AS vs *dw/dw*, $P<0.01$) [304]. Interestingly, in pregnancy, there appears to be no significant change in AS pituitary PRL content, when compared to non-pregnant AS rats. Dwarf rats showed a mean reduction in PRL content from (29.8±4.0) in non-pregnant, to (21.3±2.4) in pregnant rats, and although this remained higher (21.7±2.4) than in pregnant AS rats (14.4±2.1), the difference was no longer statistically significant. This further suggests that the dwarf rat model has an unusual increased pituitary PRL content, but that it isn't maternally altered during pregnancy, remaining higher than in AS rats. Of course these are only content measures, so it is possible there are differential effects as secretion vs release in dwarf rats. Since the mechanism for increased PRL is unclear in dwarfs, it is difficult to speculate on mechanisms for this in pregnancy in rats.

I also tested whether this high pituitary PRL content in pregnant and non-pregnant dwarfs would be affected by fasting during pregnancy. Pregnant 48 hour fasted AS rats showed no significant difference in pituitary PRL content, when compared to non-pregnant or non-fasted pregnant AS rats. Pregnant fasted dwarf rats, showed higher pituitary PRL content, than non fasted pregnant dwarf rats though the contents didn't exceed those in non-pregnant dwarfs, and pituitary PRL content in pregnant

fasted dwarfs was much higher (29.3±2.9), compared to pregnant fasted AS rats (13.4±1.9). These results indicate that the high PRL content recorded for dwarf rats is maintained during conditions of fasting, compared to non-fasted and normal rats.

Figure (3.5) Pituitary PRL content measured for AS and dwarf non-pregnant and pregnant rats.



*ANOVA followed by Student Newman Keuls, *** P<0.001*

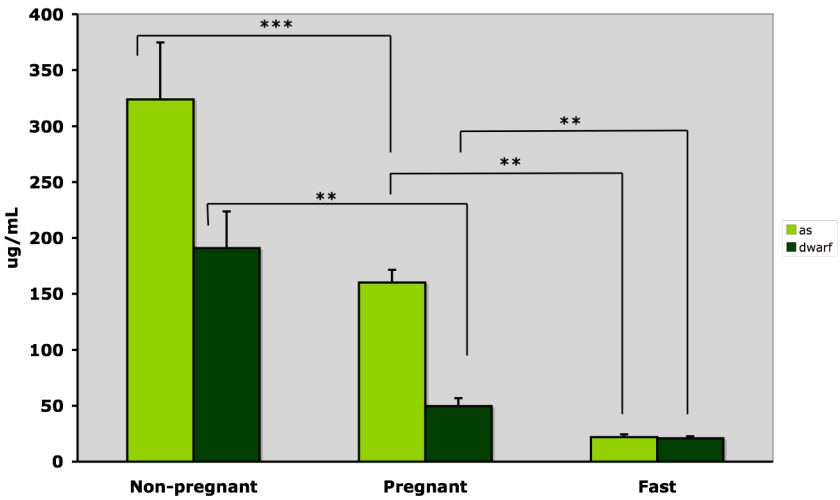
3.6.2 IGF-1 plasma levels in dwarf and AS pregnant rats during pregnancy

Circulating IGF-1 concentrations change in pregnancy in a variety of species.

In women, maternal serum levels of IGF-1 rise progressively throughout pregnancy, [250, 310] and its levels are associated with mean levels of GH-V. In contrast, there are several studies that show in rats that IGF-1 levels continue to decrease during the later part of pregnancy [173, 311, 312]. Gargosky *et al* (1994) [173] showed that maternal rat serum IGF-1 levels increased from early to mid-pregnancy, after which IGF-1 levels declined. Conversely, as IGF-1 levels declined, pituitary rat GH increased two-fold. This coincides with a decrease in IGF binding protein 3 (IGFBP-3) and the appearance of an IGFBP protease, which cleaves BP3, increasing free IGF-

1 levels. IGF-1 plasma levels are low in dwarf rats, but I measured IGF-1 levels in dwarf (and AS) rat plasma in pregnancy, as these has not previously been compared. IGF-1 plasma levels measured in dwarfs and AS rats are shown in Figure 3.6. As expected non-pregnant dwarf rats showed much lower plasma IGF-1 levels (160.0±11.3) than non-pregnant normal rats (323.9±50.8). Both 16d pregnant dwarf and AS rats showed a significant decrease of IGF-1 plasma levels, when compared to non-pregnant rats, (49.7±7.0 and 160.0±11.3 compared to 191.0±32.7 and 323±50.8; both $P<0.01$), respectively. As expected, there was also a significant decrease in IGF-1 plasma levels measured in both pregnant fasted AS and dwarf rats, compared to non-fasted counterparts (21.9±2.3 and 20.5±1.7 compared to 160.0±11.3 and 49.7±7; both $P<0.01$, respectively). The reduction in IGF-1 plasma levels shown in the fasted rats support the previous findings of Bornfeldt *et al* (1989) [161] which also showed a reduction in IGF-1 levels in fasted rats. My data shows this is also the case in food deprived pregnant rats, and in the dwarf strain.

Figure (3.6) IGF-1 plasma levels measured in AS and dwarf, non-pregnant and pregnant rats.



ANOVA followed by Student Newman Keuls, ***P<0.001, ** P<0.01

The decline in IGF-1 levels in fasting is thought to be due to, at least in part, to a lower hepatic synthesis of IGF-1 [313]. High levels of IGF-1 are known to inhibit pituitary GH synthesis and secretion by acting on the sommatotrophs [314]. Therefore, it can be expected that diminished negative feedback as a consequence of decreased IGF-1 may contribute to the increase of plasma GH during late pregnancy. This of course will be much more pronounced in normal rats than in dwarf rats, which are already IGF-1 deficient, but which cannot increase their GH levels significantly.

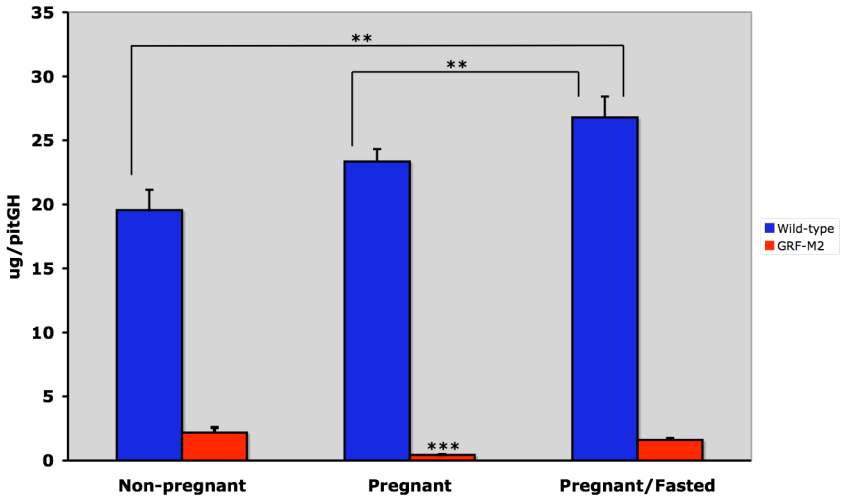
3.7 Measurement of pituitary GH, PRL content and IGF-1 plasma levels in GHD (GRF-M2) and non-transgenic pregnant and non-pregnant mice.

There have been few studies that have measured changes in the hormone content of the pituitary in mice and, none adequately monitoring plasma levels during pregnancy. The latter is not surprising considering the size of mice and the difficulty in obtaining serial blood samples from them. I therefore wished to establish some baseline data for our GRF-M2 mouse model [286]. As well as, being able to compare the results from one rodent GH deficient model to another, these comparisons would help draw further conclusions to whether any changes observed are common to at least two deficient GHD rodent models of primary and secondary dwarfism. As described in materials and methods, GRF-M2 transgenic mice lack GHRH neurons [286] show GH deficiency, secondary to GHRH deficiency, and develop dwarfism post weaning.

I confirmed that GRF-M2 mice show dramatically lower pituitary GH contents (2.2μg/pit), when compared to their non-transgenic counterparts (19.6μg/pit), Figure 3.7. Also apparent is a trend in slightly higher pituitary GH contents in pregnant non-transgenic mice, compared to a non-pregnant group (23.3±0.9 compared to 19.5±1.5)

but this did not reach statistical significance. Interestingly, pregnant GRF-M2 mice showed a significantly *lower* (0.41±0.09) pituitary GH, when compared to non-pregnant mice (2.1±0.4), P<0.001. Given this lack of GHRH, one possibility is that they are unable to respond to increased demand for pituitary GH during pregnancy by increased GHRH drive to stimulate GH synthesis. As in the GHD rat model, I was interested to see whether fasting during pregnancy would change the pituitary GH content in GHD mice or whether this change would differ in the mouse, compared to the rat. The pituitary GH content measured for pregnant wild-type mice fasted for 48 hours was obviously much higher (26.1±1.6), compared to GRF-M2 pregnant fasted mice (2.1±0.4). However, significantly higher pituitary GH contents were found in both pregnant non-transgenic and GRF-M2 fasted mice, compared to pregnant non-fasted groups (26.1±1.6 and 2.1±0.4, compared to 19.5±1.5 and 1.6±0.1, P<0.01 and P<0.001, respectively). Fasting in these animals may have more indirect effects on GH. This is suggested by the increase recorded in the pituitary contents of both groups of fasted mice. One possible mechanism may be a reduced feedback of IGF-1 during fasting conditions, thus resulting in the increase of pituitary GH content as IGF-1 has direct negative effects on GH synthesis, and wouldn't regulate an intact GHRH axis to regulate GH contents. It is also important to bear in mind that the GRF-M2 mice have secondary GH deficiency, unlike dwarf rats, therefore their pituitary somatotroph population has no intrinsic defect, and should be responsive to feedback mechanisms, providing it excludes the requirement of increased GRF, which is lacking in these GRF-M2 mice.

Figure (3.7) Pituitary GH content measured for non-transgenic (wild-type) and GRF-M2 non-pregnant and pregnant mice.



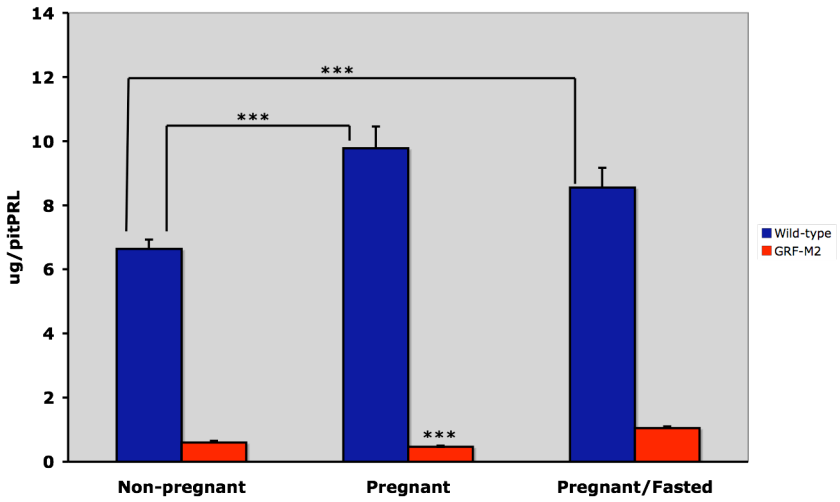
*ANOVA followed by student Newman Keuls, **P<0.01, or ***P<0.001.*

3.7.1 Pituitary PRL content measured in non-transgenic and GRF-M2 non-pregnant and pregnant mice

Unlike the dwarf rats, and like most GHD models, GRF-M2 mice show low PRL in the non-pregnant state [286], so it was interesting to find out whether there was be an increase in PRL during pregnancy. Figure 3.8 confirms the amount of pituitary PRL measured for GRF-M2 mice was markedly lower (0.6±0.03) than that in non-transgenic mice (6.6±02), similar to the pituitary GH comparisons (Fig 3.8 vs Fig 3.7). A significantly increased pituitary PRL content was observed in pregnant non-transgenic mice (9.7±06), compared to the non-pregnant group (6.6±0.2, P<0.001). This differed from my results in normal rats, in which no significant difference in pituitary PRL levels was observed between any groups. Unlike their wild-type littermates, GRF-M2 pregnant mice showed a further reduction in PRL-pituitary content, when compared to non-pregnant mice (0.61±0.03 compared to 0.42±0.01,

P<0.001). This supports the paper describing the GRF-M2 model, in which the potential mechanism for disruption of lactotrophs (secondary to GH loss) is discussed [286]. Figure 3.8 also shows that fasted pregnant non-transgenic mice have higher pituitary PRL contents (8.5±0.6), compared to the GRF-M2 group (1.05±0.03), which in turn was higher than PRL contents in the non-fasted pregnant group (0.42±0.01, P<0.001).

Figure (3.8) Pituitary PRL content measured for non-transgenic (WT) and GRF-M2 non-pregnant and pregnant mice.



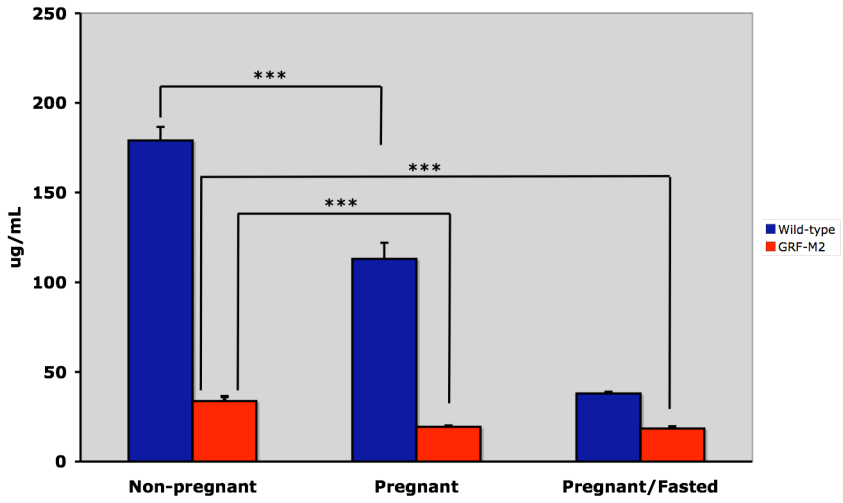
*ANOVA followed by student Newman Keuls, ***P<0.001.*

3.7.2 IGF-1 plasma levels measured in GRF-M2 and wild type mice during pregnancy

As in dwarf and AS rats, IGF-1 plasma levels (Fig 3.9) are significantly reduced in the pregnant GRF-M2 and non-transgenic mice, when compared to non-pregnant groups, (19.3±0.4, 113.0±8.9 compared to 33.7±2.5 and 179.1±7.5, both P<0.001,

respectively). Figure 4 also shows a significant reduction of IGF-1 plasma levels in the fasted pregnant GRF-M2 group (18.4±0.9) compared to non-pregnant mice (33.7±2.5, P<0.001). This adds strength to the notion that GHD in pregnant rodents show reduced IGF-1 production, unlike in humans.

Figure (3.9) IGF-1 plasma levels measured in GRF-M2 and non-transgenic (Wild type) non-pregnant and pregnant mice



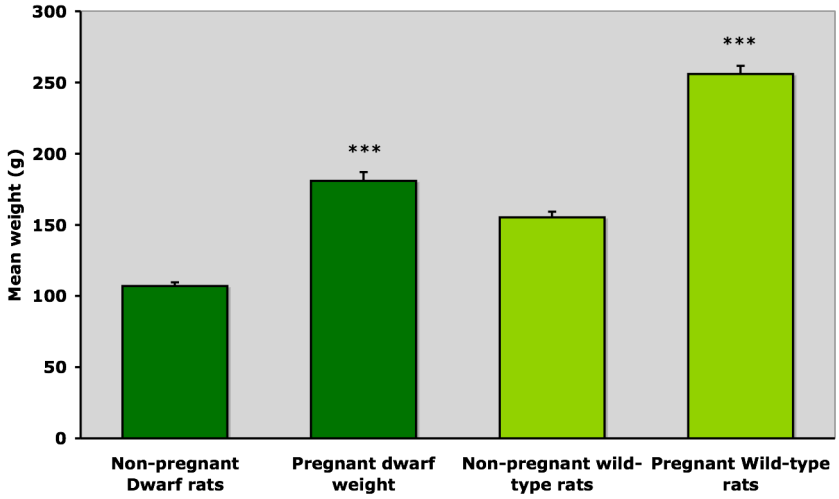
ANOVA followed by student Newman Keuls, ***P<0.001.

3.8 Difference in weight gain observed in pregnant and non-pregnant GHD rodents and normal Wild-Type controls

Figure 3.10 shows the weights recorded for day (16) pregnant vs non-pregnant dwarf and AS rats. Figure 3.10 shows that, non-pregnant dwarf rats weigh one third less (107.2g±2.55) than to non-pregnant AS rats (155.0g±4.03; P<0.001), in line with observations made by Charlton *et al* (1988) [300]. Figure 6 also shows that this relative dwarfism persists in pregnancy, since pregnant dwarf rats weighed

significantly less (180.3g±6.25) than pregnant AS rats (255.0g±5.88; P<0001). However, it was interesting to see whether pregnant dwarf rats gained less weight than normal pregnant AS rats, e.g. by expressing this relative weight gain as a percentage. Dwarf rats gained 40% weight during the 16 days of pregnancy, whilst AS rat's weights increased by 39%, i.e. identical between dwarf and AS rats. Neglecting for the moment, pup weight and number, the overall weight gain of mothers would differ if the GH-deficient mothers tissues continue to be affected by GH deficiency as would be expected from my hormone data.

Figure (3.10) The weight of non-pregnant and pregnant dwarf and AS rats

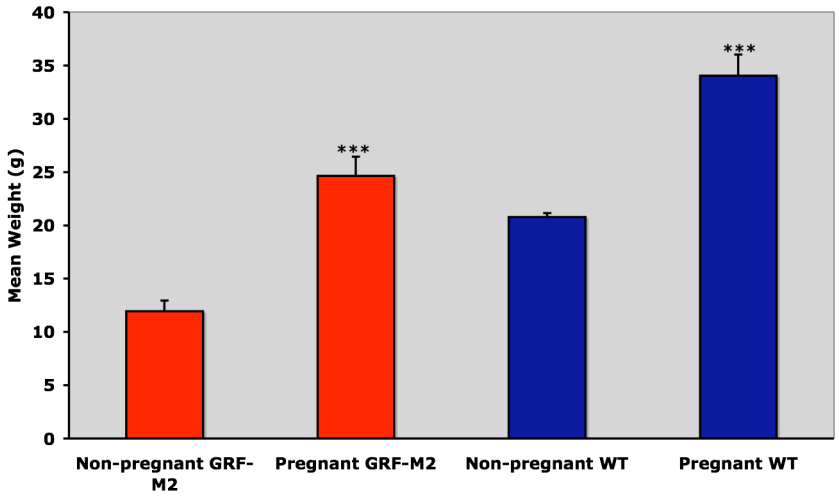


Student t-test, ***P<0.001

The same measurements were made in 16 day pregnant GH-deficient, GRF-M2 and non-transgenic mice. Figure 3.11 shows an increase in the weight of pregnant GRF-M2 and non-transgenic mice (24.3g±1.8 and 34.0g±1.8) compared to non-pregnant groups (11.0g ±1.0 and 20.1g±0.4, both P<0.001). As in the dwarf rats, GRF-M2 mice show post weaning dwarfism; non-pregnant female GRF-M2 mice weighed

13.8g±0.5, compared to non-transgenic females 23.1g±1.8 [286]. However, GRF-M2 mice show a pregnancy related percentage weight gain of 54% relative to its weight, compared to 41% weight gain showed by the non-transgenic mice. This indicates that the GRF-M2 mice do not show any apparent impairment in weight gain during pregnancy, gaining over half of their relative body weight. Of course this does not distinguish between pup weight and litter number, vs maternal growth, and the former could have a relatively larger numerical effect in these tiny GRF-M2 mothers.

Figure (3.11) Weights of pregnant and non-pregnant GHD (GRF-M2) and non-transgenic wild-type mice.



Student t-test, ***P<0.001

3.8.1 Differences observed in the number of pups born to normal vs GHD pregnant rodents

Previous work has shown that GH may have a role in determining litter size. A study using GH receptor knockout mice showed a marked reduction in the litter size born to these animals. GHR KO mice showed a reduction in litter sizes in GHR KO

homozygous mating (1.6±0.4 pups per litter vs. 6.3±0.9 for wild-type mating vs. 3.2±0.5 for heterozygous mating), but there are many possible reasons for this, not least being strain differences [315]. It was therefore interesting to determine whether similar effects on the number of pups per litter would be seen in the GH-deficient models used in my experiments, when compared to normal rodents of the same strain. Figure 3.12 records the number of pups observed for 40 individual litters in dwarf and AS rats. The most frequently occurring litter size in dwarfs was 8, compared to 10 in AS rats, on the same genetic background. Figure 3.13 shows a similar scatter graph for GRF-M2 and non-transgenic mice, revealing a reduced average litter size in GRF-M2 mice, most frequently 5, compared to 10 in non-transgenic mice. These results, showing that a reduced number of pups are born to both GRF-M2 and dwarf rats, compared to normal rodents, do suggest that GH-deficiency and dwarfism impairs litter size, and this will exaggerate the differences observed earlier in total weight gain.

It is difficult to determine whether the absence of GH is directly responsible for the reduction of pups per litter or this reflects a secondary phenotype of dwarfisim. Nevertheless, there is data in the literature, which shows maternal GH to be involved in oocyte maturation, ovulation rate and folliculogenesis [316, 317]. It is thus possible that GH may have a possible role in determining litter size. (see page 110 for discussion).

Figure (3.12) The number of pups born per litter in dwarf and normal AS rats.

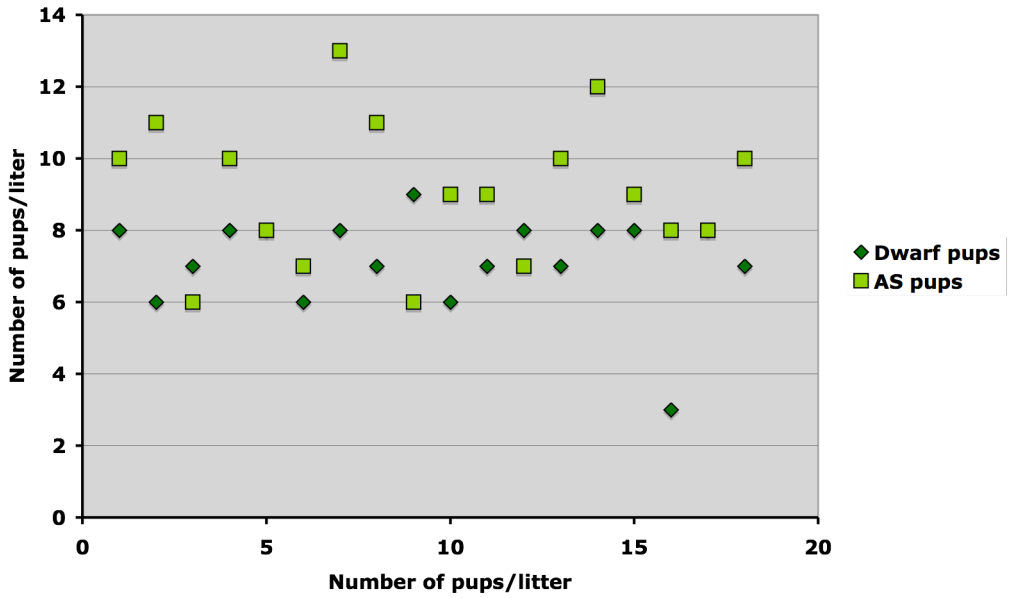
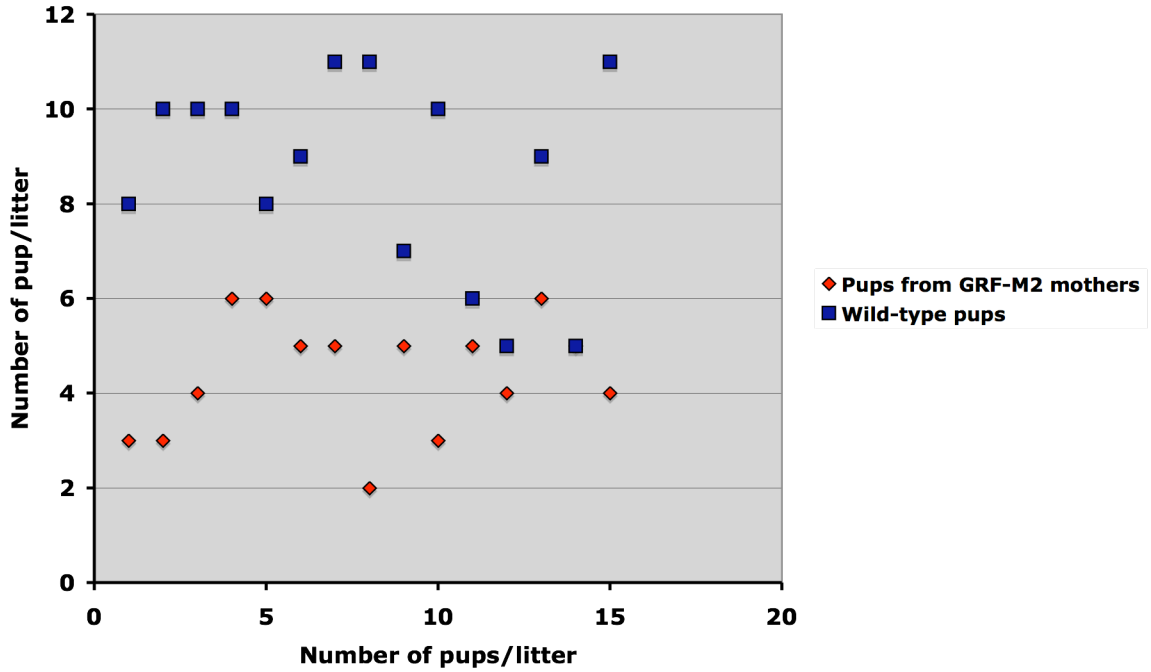


Figure (3.13) The number of pups born per litter to GRF-M2 mice and normal Wild-Type mice.



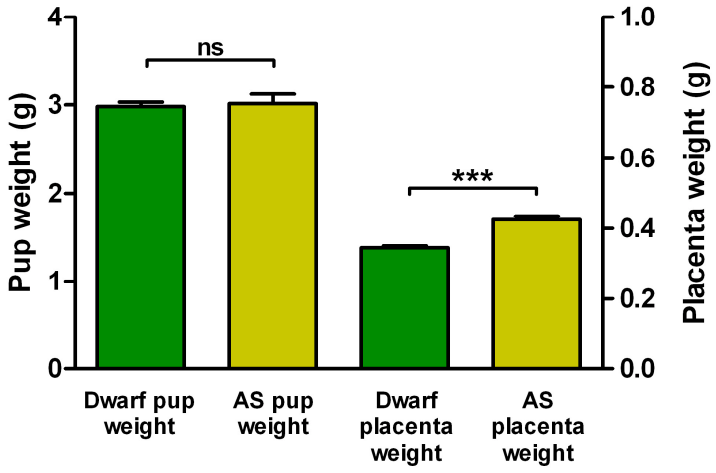
3.8.2 Differences in the weight of pups and placenta in GHD vs normal rodents

Unlike littersize effects, Figure 3.14 shows no significant difference in the weight of individuals pups born to dwarfs, compared to that of AS rats, which further supports the idea that most of the difference in maternal weight gain is due to effects in the mother, and on total litter size.

Although, no study has shown the direct affects of maternal GH on fetal growth, a study by Spencer *et al* (1994) [318], shows that disruption of rat GH during pregnancy can lead to the retardation of fetal and placental weights, suggesting that the effects of GH during pregnancy are more likely to be indirect. Furthermore, GH treatment throughout pregnancy of GHD rats, in the same study, resulted in significantly bigger pups, compared to pups from untreated mothers. In contrast, in humans lack of GH-receptors causing complete GH insensitivity (as in Larons syndrome), is not associated with any changes in birth weight [319] suggesting that GH signaling is not important for fetal growth in humans. From these observations, it appears that whilst GH-deficiency in dwarf rats somehow reduces the number of pups successfully coming to term, the weight of these pups remain similar to weights of pups born to normal rats, despite them being developed in a dwarf uterine environment. Whether this reflects a trade-off between uterine size, and nutrient flow appropriate for a smaller total litter, remains to be determined.

In this context, Figure 3.14 showed that there is a significant reduction in the individual placental weights measured in dwarfs, compared to AS rats (0.34±0.06 compared to 0.43±0.09; P<0.001). There are very few studies related to GH status and placental weight, though a relationship between rodent placental size and IGF-1 levels, has been reported [224]. McIntyre *et al* [224] showed in rats that reduced IGF-1 plasma levels during pregnancy, were associated with reductions in placental size. Since plasma IGF-1, in pregnant dwarf rats in my experiments, also showed a decline compared to non-pregnant rats, this is consistent with an indirect effect of GHD on placental weight, via lower IGF-1 levels.

Figure (3.14) Pup and placental weights in dwarf and AS rats.



******P<0.001, student t-test.***

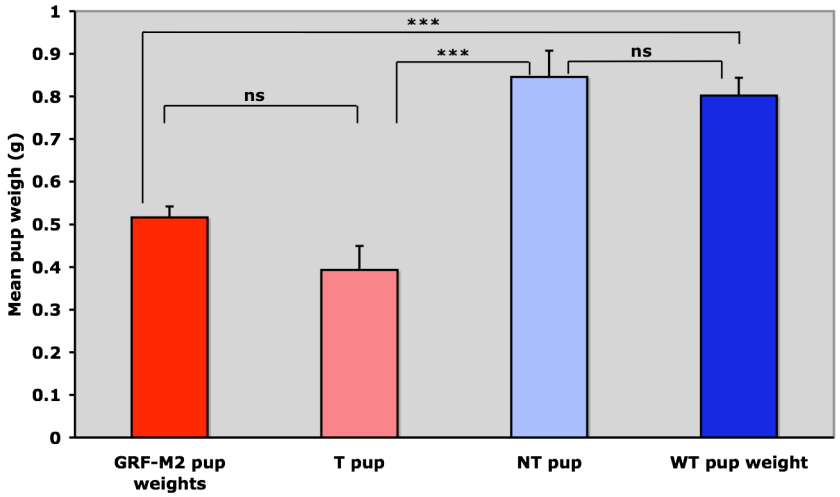
It was also interesting to test whether pup and placental weights varied in the same way in pregnant normal and GHD *mice*. However, in addition, in these experiments, pups from these litters were also individually genotyped to record whether there was any difference in weight between the transgenic and non-transgenic pups, carried in the same mother. This was possible in mice, as the phenotype is expressed in hemizygous animals, (whereas the rat *dw/dw* mutation is recessive, heterozygotes being normal, and there is no genetic PCR maker for *dw/dw*). Figure 3.15 shows that the mean pup weights recorded for GRF-M2 litters were significantly smaller (0.51±0.03), than the mean pup weights in wild-type litters (0.80±0.04, $P<0.001$). The difference in mean pup weights between transgenic and normal wild type pups suggests in mice, that the mothers GHD-status may play a part in determining the weight of its pups, however, the mechanism involved is unclear. Again, it is quite possible that this simply reflects constraint imposed by the smaller uterus, as well as the reduced levels of IGF-1, which in turn may limit the nutritional supply to pups.

Figure 3.15 shows that there is a significant reduction in the mean weight of transgenic pups, compared to non-transgenic pups born to the same litter (0.393±0.05 compared to 0.84±0.06; $P<0.001$, respectively), but no significant difference between the weights of non-transgenic pups, compared to wild type pups. These results suggest that the absence of maternal GH doesn't have a direct effect on fetal growth, as non-transgenic pups from the same litter as transgenic pups, grow normally, when compared to normal pups born to normal mothers, it does raise the surprising notion that the lack of GHRH may have some effect on the transgenic pups in utero. Since GH does not seem to play a role in the fetus, per se, it may point to other effects of the GHRH gene in fetal life.

Interestingly, despite these differences in pup weights the mean placental weights recorded for both GRF-M2 and wild-type litters were not different (Figure 3.16), nor were there significant differences in mean placental weight from transgenic vs from non-transgenic pups (Figure 3.16). The study describing the GRF-M2 mouse mentions the potential for the expression of GRF-M2 ablating transgene in the mouse placenta, which silenced and ablated virtually all GHRH cells in the hypothalamus [286].

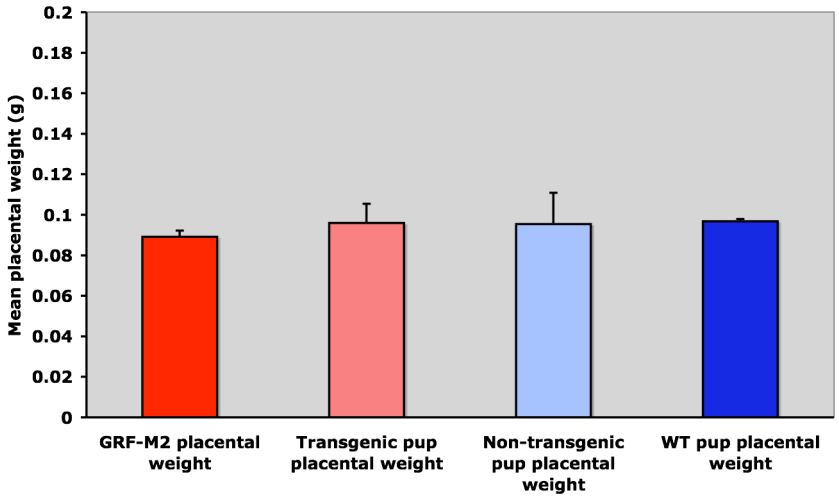
No defects were observed in this study, suggesting that (i) the mouse placenta isn't affected directly by this model of secondary GH deficiency, and (ii) any GHRH release from the placenta probably doesn't affect pituitary GH secretion. Furthermore, no correlation was observed with pup GH-status and placental weight for either transgenic or non-transgenic pups (data not shown). Nevertheless the effect of genotype on fetal weight remains to be explained, and the most economical hypothesis is that there may be adverse effects in individual pups carrying the M2 transgene.

Figure (3.15) Pup weights recorded for GRF-M2 and wild-type litters, as well as (genotyped) transgenic and non-transgenic pups



*Pup weights recorded for GRF-M2 litters are shown in the (red) bar, the light red bar shows the weights of pups of which are transgenic from the same litters, as the light blue bar shows the weight of those that are non-transgenic. The pup weights of non-transgenic (WT) pups are shown in the (blue) bar, ANOVA followed by student Newman keels test, ***P<0.001.*

Figure (3.16) Placental weights recorded for GRF-M2 and wild-type litters, as well as placental weights for pups known to be transgenic or non-transgenic.



The color code follows the same format as above, except in this case the weights are shown for placenta for individual mice

3.9 Discussion

Unsurprisingly, pituitary GH contents in both dwarf rats and GRF-M2 mice were confirmed to be significantly lower, compared to normal rodents. However, my new observations were the measurements of pituitary GH contents in both models during pregnancy. Neither the dwarf rats nor the normal rats showed changes in pituitary GH content, during pregnancy, and they remained low, when compared to normal rats.

The number of papers that have recorded endogenous GH plasma levels during rat pregnancy is scarce, however, Carlsson *et al* (1990) [81] showed the plasma GH levels increased with the progression of pregnancy. Since I only measured GH in the pituitary, lack of change in the pituitary content does not necessarily imply there to be no change in serum levels, for which I would need to perform serial micro sampling. It is thus possible that both synthesis and secretion increased with no overall change in content, but this rarely occurs in experiments where others have previously measured both in my lab (Robinson, personal communication). Such information is only available for the rat, as serial sampling is rarely performed in mice.

The GRF-M2 model as described before has GHRH neurons ablated, using the modified H37A variant of the influenza virus M2 protein expressed in these neurons [286]. A few GH cells remain in these mice but the anterior pituitary shows marked hypoplasia, with greatly reduced GH content and reduced release of GH.

During pregnancy an increased secretory pattern of GH might also be expected in mice. In these GRF-M2 mice, disruption of GHRH neurons would result in such an increased demand not being met. The further reduction in pituitary GH content observed in pregnancy could be a result of non-hypothalamic mechanisms, such as

changes in IGF-1 affecting GH synthesis in these mice. It should also be remembered that the somatotrophs in these mice (unlike in dwarf rats) have no intrinsic defects, so would normally respond to other secretagogues and metabolic factors. Pituitary prolactin contents measured for both GH-deficient rodent models produced interesting contrasting observations. The dwarf rats showed a higher significant pituitary prolactin content, compared to normal (AS) rats. This observation confirmed previous reports that dwarf rats have elevated levels of pituitary prolactin, compared to normal rats [305, 306]. With the combination of immunocytochemistry and fluorescence-activated cell sorting [309], Tierney *et al* measured GH-PRL cell numbers in dwarf rats and despite the evident somatotroph hypoplasia, they showed an increase in lactotroph number and increased prolactin stores. It was therefore interesting to find in my studies that the pituitary prolactin content during pregnancy decreased compared to non-pregnant rats in both dwarf and AS strains.

Escalada *et al* (1996) [320] showed that early pregnancy in normal rats is associated with high prolactin plasma levels and low pituitary prolactin content, but this reverses in late pregnancy. My observations taken at day 16 of both rat and mouse pregnancy may not support these findings, but to accurately show the changes in pituitary prolactin levels in dwarf rats, both plasma and pituitary prolactin levels would need to be observed throughout pregnancy, and then compared to normal rats. I did not follow this up as it wasn't important for the main aim of my work, especially as the GRF-M2 mouse showed in contrast to the dwarf rat, a reduction in pituitary prolactin content in both non-pregnant and pregnant mice, when compared to non-transgenic animals.
This was no surprise as Le Tissier *et al* (2005) [286] has previously reported prolactin deficiency in the GRF-M2 model, like in most other model of somatotroph hypoplasia

[321] as GH cells share a common lineage with prolactin cells. One possibility arising from my data is that PRL cells could be differentially sensitive to low IGF-1 levels in these two models of primary and secondary GHD.

Of more relevance for GH secretion, IGF-1 serum levels in both pregnant dwarf rats and GRF-M2 mice were reduced when compared to non-pregnant counterparts. This is in contrast to what happens in human pregnancy, in which IGF-1 plasma levels are shown to increase with the increase of placental GH [115]. However, it is in agreement with other authors [173, 311]. A possible cause of the decline in plasma IGF-1 levels could be a result of a reduction in IGF-1 mRNA levels driven by GH in the liver [311] and a further contribution may be the consequence of a decrease in GH-dependent IGFBP-3 [322] leading to the decrease in the half-life of serum IGF-1. Another possibility could be an increased insulin resistance in pregnancy, lowering hepatic IGF-1 production. The reduction of IGF-1 levels could also offer an explanation to the increase of GH plasma levels in rat pregnancy. Circulating IGF-1 inhibits pituitary GH synthesis and secretion by somatotrophs [314] so diminished negative feedback from decreased IGF-1 levels, may contribute to the increase of plasma GH synthesis and release during late pregnancy.

Both dwarf rats and GRF-M2 mice gained similar amounts of weight as normal pregnant rodents, with both dwarf rats and GRF-M2 mice showing almost doubling of their total weight during pregnancy. In both dwarf rats and GRF-M2 mice, there were a reduced number of pups born per litter. This also suggests a likely indirect effect of the mothers GH-deficiency in affecting the number of pups per pregnancy. There is evidence from others to suggest that the lack of GH and functioning GH-receptor can

affect oocyte maturation and ovulation rate, which in turn, alter the number of pups per litter in various species. GHRs are present in ovarian granulosa cells, corpus lutea and oocytes in rats [323] and humans [324]. GH is able to induce ovarian IGF-1 synthesis and to increase granulosa cell LH receptor number [316]. GH treatment increases the number of mature follicles in the rat [316] and rabbit [325] largely as a result of decreased apoptosis and atresia of preantral follicles [326] increased granulosa cell estradiol production [327] and the gonadotrophin potentiating actions of IGF-1.

GH is also known to have substantial stimulatory effect on oocyte maturation *in vitro*, in the cow [328], rat [329], rabbit [325], and human [330] this occurs with the accelerated breakdown of the germinal vesicle and accelerated passage to metaphase II. This may be a direct action of GH, mediated by local IGF-1, since germinal vesicle and metaphase II oocytes express GHR mRNA [331]. Moreover, there may be other factors independent from number of oocytes, which may contribute to determining the number of pups per litter. GH may also play a role in modulating endometrial secretion and receptivity, since GH-receptors have been reported in endometrial glands and have been shown to increase in gland density in response to maternal GH [332]

There was no significant difference in the weight of pups born to dwarf rats, compared to those born to normal rats, suggesting that the GH-deficiency of the mother or the presence of the mutation in the fetus does not play a major role in regulating the weight of the pups directly. Laron dwarfs with inactivating GHR mutations are born slightly (up to 2 SD) shorter then normal [99] and congenitally

GH- deficient newborn babies are also shorter [298], however, their birth weights are usually normal. Unfortunately my observations didn't include measurement of the length of the pups, which could have shed light on these differences. Note also that these results differed in mice; the weight of pups born to GRF-M2 mice were shown to be significantly smaller, compared to normal mice, and when genotyping was performed, the transgenic pups alone showed a significant reduction of weight. Thus, in contrast to my findings in the rat, these results suggest that the maternal GH status or transgene expression in the fetus can exert greater effect on birth weight. A study by Danilovich *et al* (1999) [315] suggests that the fetal length and weight of GHR KO mice are reduced by 20-25% at E17, furthermore, weight at birth being reduced by 17%. I need to bear in mind that the mechanism of GHD is indirect in my mouse model, and cannot exclude a role of GHRH itself.

How maternal GH may influence fetal growth and birth weight indirectly is far from clear, however, some studies suggest that maternal GH may regulate the availability of substrates to the fetus [32]. Maternal GH may have an influence on birth weight through its regulatory actions to make glucose, available as a metabolic substrate for the fetus [333]. However, my data are informative since the pups that showed reduction in birth weight in GRF-M2 mice were only those that were genotyped to be transgenic, suggesting an effect in the fetus itself. The reduction in the size of the transgenic pup could reflect its ability to utilize its substrate supply, and it would be difficult to imagine how the supply of important substrates could be selective between pups, depending on genotype. However, if the supplier of substrates to each pup, the placenta, was also globally affected by the GH-status of the mother, then perhaps the amount of substrates being extracted by each pup would be different and an

assortment of birth weights could be seen. However, it doesn't appear to be simply due to differing placental weights, as placental weights from GRF-M2 mice were no different to those recorded for normal mice. Another speculation would be an effect mediated on placental GHRH mediated via the transgene selectively in the fetus.

Dwarf rats also showed a significant reduction in placental weights compared to normal rats. The placenta is comprised of both maternal and embryonic tissues, therefore a difference in maternal GH-status could affect this contribution to the placenta and thus influence the process of fetal growth. However, despite differences in placental weight, the dwarf rat pup weights were within normal ranges. The conflicting findings of differences in placental weights between rats and mice, makes it difficult to draw any general conclusions from my studies, other then to record that GHD can have significant effects on the feto-placental unit in rodents. I found reduced IGF-1 levels in these models, confirming previous findings. This provides an easy explanation for the increased GH plasma levels reported in normal pregnant rats, but not in GHD rats, who cannot respond further. Although IGF-1 levels are lower in rodent pregnancy, IGF-1 still seems the most likely candidate to be mediating the indirect effects of maternal GH in fetal growth, given that manipulation of IGF expression in mice and/or signals has been used to establish the role of the IGFs in feto-placental growth (reviewed by Esfstratiadis, 1998). Deletion of either the IGF-1 or IGF-2 gene in mice results in retardation in fetal growth to similar extents, with a 40% reduction in birth weight compared to wild-type littermates [177, 334]. Furthermore, double knockouts of both IGF-1 and IGF-2 exhibit an additive growth retardation of 80%. Disruption of the IGF-1 receptor in mice also leads to a 55% decrease in birth weight, which is less than in the double (IGF-1/IGF-2) knockouts,

[335]. The additional loss of IGF-1 in IGF-IR knockouts does not appear to show any further decrease in birth weight, suggesting that IGF-1 functions solely via the IGF-1R. Finally, IGF-2 and IGF-1R knockouts show greater growth retardation than in single IGF-1R knockouts, suggesting that IGF-2 functions via another receptor during fetal growth [336]. Interestingly, in mice, deletion of the IGF-2 gene has more severe effects on the placenta, resulting in a 40% reduction in size, compared to deletions of the IGF-1 or IGF-1R [335].

Placental growth in mice is normal in the absence of both IGF-1R and insulin receptor, suggesting that IGF-2 may, as mentioned above, act through a unknown placental specific receptor [178]. Taken together it appears that IGF-1 affects fetal growth directly, whereas the growth promoting actions of IGF-2 maybe indirectly mediated via changes in the growth and nutrient transfer in the placenta, [178]. Direct administration of IGF-1 to sheep and monkeys show an increase in the weight of specific fetal organs as well as skeletal maturation [337] suggesting the direct anabolic actions of IGF-1 on fetal metabolism. Nutrient restriction also shows a more pronounced effect on circulating levels of IGF-1 than IGF-2, similarly, there is a greater reduction in tissue abundance of IGF-1 than IGF-2 mRNA during nutrient restriction in fetal rats and sheep [338, 339]. These observations are consistent with the findings that birth weight is more closely correlated with plasma IGF-1 than IGF-2 in several species [340]. Reports of rare case studies in IGF-1 deficiency in humans have shown phenotypes of severe intrauterine growth restriction as well as mental retardation [341, 342]. Furthermore, cases of IGF receptor deletions in children have also reported similar severe pre and post-natal growth retardation [343, 344].

Collectively, these findings support the importance of IGFs during fetal growth with different roles for the IGFs in bringing about their growth stimulatory effects.

In summary, the effects of having a GH deficient mother is far from understood but my observations in the reduction of number of pups per litter and the weight of pups suggest some indirect influence. However, the underlying mechanism, the involvement of other factors such as IGFs, and their autocrine/paracrine effects in these GHD rodent models during pregnancy remain to be elucidated.

4. Is the placenta a direct novel target for growth hormone?

4.1 Introduction

In chapter one I described the known target tissues for growth hormone (GH), and the evidence for the indirect effects of GH on growth and metabolism, through the local generation of IGF-1. The aim of the experiments described in this chapter is to test whether the placenta could be a direct target organ for GH. Moreover, it could be an autocrine or paracrine target in humans as the human produces a placental form of GH, growth hormone variant (GH-V) [202, 205]. The same placental form of GH is not produced during pregnancy in rodents, but there are marked similarities in the pattern of secretion of placental GH in humans and pituitary GH (GH-N) in rodents during pregnancy, suggesting that the same endocrine effect (increased continuous GH exposure) may be achieved by different means [2, 81]. Although the mechanisms appear different (pituitary pattern in rodents, continuous placental production in primates) in both cases, the end result is a switch from pulsatile to continuous GH exposure in pregnancy. I feel it reasonable to speculate that the switch in the pattern of GH secretion, from episodic to more continuous may be of some importance during pregnancy, rather than just the a change in source of the GH production, and that the role of continuous exposure, either of GH-V or GH-N may be common to several different species. However, this begs the question of what is the important target for continuous GH. Recent methodological developments have made it possible to identify target tissues responding directly to GH. The aim of this chapter is to use these methods to test whether the placenta could be a direct target for GH, using GH deficient rodent models which lack pituitary GH, and in which the placenta is not a source of GH-V, so that exogenous GH could be given.

There is good circumstantial evidence to believe that GH may act directly on the placenta. Firstly, GH-receptor (GHR) is expressed in the placenta and several studies have shown an increase in expression of GHR and growth hormone binding protein (GHBP) in both the liver and placenta of rodents throughout pregnancy [345, 346]. Secondly, placental human cell lines have been reported to be responsive to GH [283] though I found that difficult to reproduce. In the experiments described in this chapter, I used GH-deficient rats and mice (as described in methods), and a newly developed method to visualize the direct actions of GH on the placenta. For a control known direct target tissue for GH, I also studied the responses in maternal liver, which might also play an important role responding to placental GH, as it does to pituitary GH.

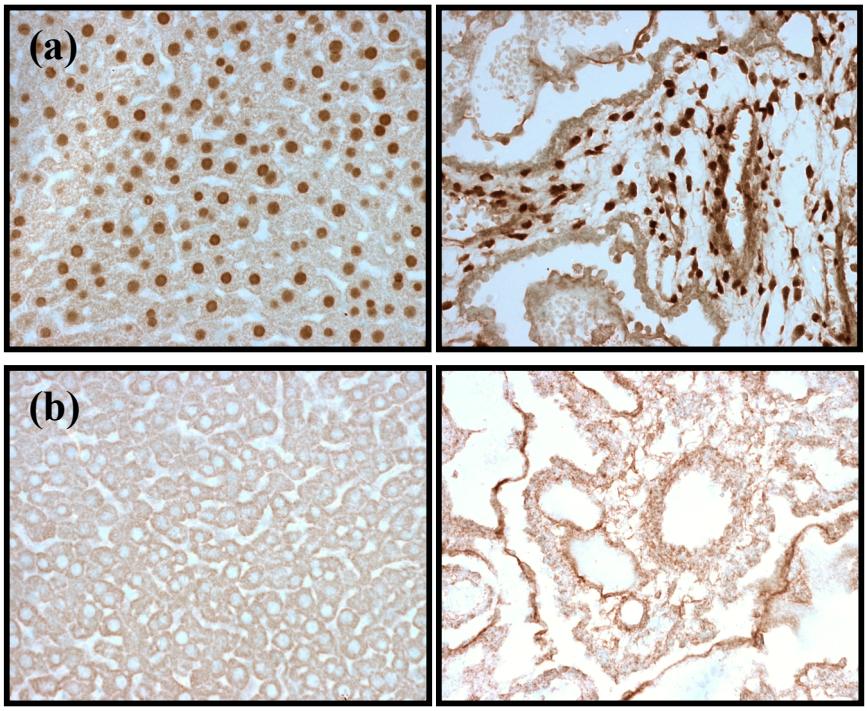
As reviewed in chapter 1, GH-GHR interaction causes a number of phosphorylation events mediated via JAK2, resulting in the phosphorylation of signal transducers and activators of transcription (STATs). Recent papers by Rui *et al* (2008) [291] have exploited histochemical methods to show that cells in GH target tissues are responsive to a single injection of GH in GH-deficient animal models. In these studies, direct action of the GH signal transduction cascade was visualized by detecting GH-induced phosphorylation of Stat5 after a single injection of GH, at a time too soon to reflect any consequences of indirect GH action (e.g. IGF-1 generation). I used the same detection method to visualize responding cells in the placenta of pregnant rodents. It was important to use GHD animals for two reasons, firstly, because the signal is much clearer in the absence of endogenous pituitary GH, which could be secreted spontaneously, complicating the response observed. Secondly, because the method described is much more sensitive in GHD models (E.Gevers, personal communication).

4.2 Results: initial proof of concept

Two groups of six pregnant *dw/dw* rats were given a single intravenous injection of either 100μg bovine growth hormone (bGH)/ bodyweight, or saline through the tail vein. All animals were culled 25 minutes later, blood samples and tissues were harvested and the latter processed for immunohistochemistry (see Methods).

Figure 4.1 illustrates the result of staining for phosphorylated Stat5 (pYStat5) in tissue sections. In the liver (left panel), the majority of nuclei from GH-injected rats (a) showed intense staining for pYStat5, clearly different from liver sections (b) from saline-injected rats. This corresponds to the results described by Gevers [291] for non-pregnant animals, and shown that hepatic cells respond directly to GH also in pregnant animals. The right panel shows the results obtained in random placental sections from the same animals. Numerous pYStat5 positive nuclei (stained dark brown) were evident after bGH injection but not in the saline injected animals. All sections were processed at the same time, using the same antibody and experimental conditions, and was encouraging as it suggested clearly that the placenta could be a direct target for GH, at least when the hormone is given an intravenous pulse, and pYStat5 is measured.

Figure (4.1) Liver and placental sections taken from a pregnant dwarf rat injected with a single intravenous injection of bGH or saline



a) Immunohistochemistry for yPStat5 in liver and placental tissue of 16 day pregnant dwarf rats (n=6), that have received a single IV injection of bovine growth hormone, 100µg/100g body weight, 25 min before culling. b) Liver and placental sections from control animals injected with saline, and processed for Stat5 immunoreactivity. The chromogen used was DAB (diaminobenzidine), to give dark brown positive staining (see methods).

Note that the dark nuclear staining in hepatocytes (the top left of panel a, Figure 4.1) was intense and distributed quite homogenously throughout the section. In contrast, the dark nuclear staining in the right of panel a, Figure 4.1, showed distinct regions of the placental sections responded to bGH. This is the first report of such *in situ* staining, but from the location of the cells, it would appear that cells responsive to GH were mainly in the labyrinth zone of the placenta, though these were only random sections, so I could not exclude other regions that might contain responding cells in

this pilot study. The trophoblastic region of the chorionic villi consists of three types of trophoblastic cells, and is termed as trichorial, in contrast to the human placenta, which is monochorial [347]. The trophoblast layer lining the maternal blood spaces consists of mononuclear trophoblast cells (cytotrophoblast), and the middle and inner third layers are syncytiotrophoblastic. There are few antibodies available for specific trophoblast cell markers, particularly for the rodent placenta. One reason for this may be because of the multiple lineages of trophoblaslastic cells and the diversity of their location within all the regions of the rodent placenta.

4.2.1 Image Analysis

Although a positive cellular Stat5b response was obtained in pregnant rat tissues, it was quite variable in intensity. I realised I needed to develop an objective, quantitative method to analyse these stained sections in order to make statistical comparisons between responding and non-responding tissues (and saline controls) in subsequent experiments. This would also enable comparisons to be made between various experimental conditions, varying dose, pattern of bGH secretion, and the effects on the intensity of staining, and number of responding cells.

4.2.2 Analysis of immunostaining: a semi-automated approach

Histology-based results are often considered hard to quantify. A number of problems can arise which can be specific to the tissue being stained or the method being employed to visualise the staining. Using 3.3'-diaminobenzidine (DAB) as a chromogen (which creates a brown stained precipitation wherever there is secondary peroxidase labelled antibody) is a reliable and standard detection method.

However, depending on the tissue, DAB staining is particularly prone to non-specific and variable background staining, which can also vary from section to section within the same experiment. I aimed to quantify the level of response by assessing the intensity of the brown staining, as well as counting the number and proportion of cells in a responding tissue, using more objective methods than visual scoring.

The method also needed to take into account variable background staining in different tissues, which contributes noise to the specific staining signal, and also recognise potential interference from non-specific staining, which could contribute as false positive or false negative signal during any semi-automated quantification process. The quantification method I chose was part of the java-based image-processing programme, "ImageJ". I chose ImageJ as it is designed with an open architecture that can provide the extensibility to devise custom made programmes via its built in editor and Java compiler. The advantage of this is that I could obtain a package customized specifically to my needs. However, I recognise that all packages have drawbacks with how rigid or flexible they are when assessing irregular objects in images using defined parameters. I worked with NIMR's imaging software experts to develop and test a software method suitable for my images, but all the analyses were carried out by myself. What follows is a brief explanation of the process, with a series of images and worked examples, to illustrate the method in practice.

To simplify the quantification process, I decided to use some existing parameters of Image J, to collect the relevant information for each experimental image, and then calculate the information of interest. A primary need is to identify the responding (brown stained) nuclei, using a cut-off for the hue value in the pixel image of a

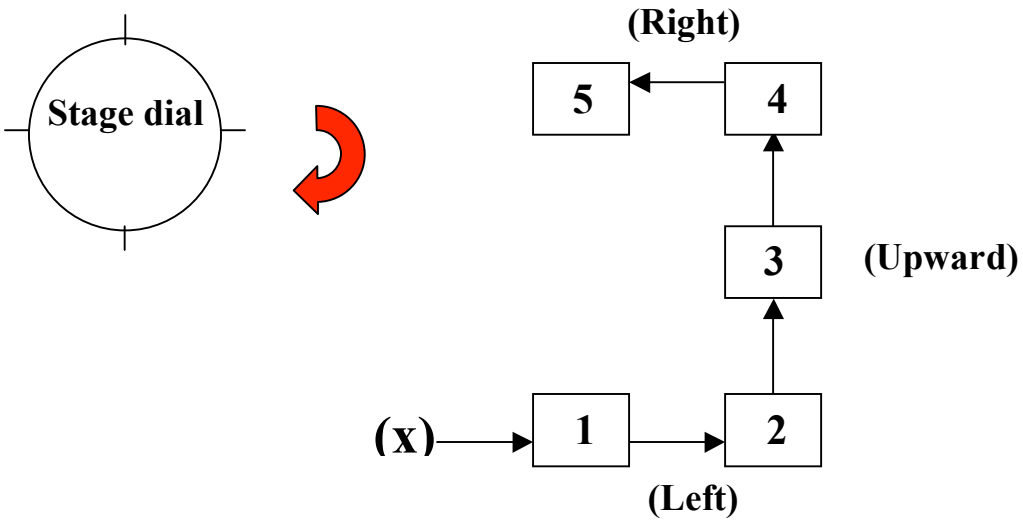
defined range of size. This meant that objects possessing a different hue value (non-specific, endogenous colour) could be discriminated and therefore not counted in the measurements. A secondary function was used to devise an estimate of the staining intensity of such identified objects (nuclei) using the "saturation" parameter in imageJ. Once the nuclei were localised in the image, they were counted in a standard grid area, so the data could then be expressed as mean number of responded nuclei and mean nuclei size (in pixels), in standard areas of section, in replicates. I set an additional parameter to allow flexibility in assessing non-specific and background staining, to minimise its input on the signals. This was achieved for each batch of sections analysed by choosing background measurements for saturation, and then subtracting them in an unbiased way from all the images, prior to analysis. It was therefore important to process batches of sections obtained stained, and processed together, before pooling results.

The calculation of the mean size of nuclei enabled a value to be set as an upper and lower threshold for nuclear size. This allowed any non-specific staining of most other objects in the field to be identified and discarded, before final calculations were made for nuclei in each image. The disadvantage of this was that "positive" cells with partial staining of nuclei (restricted to the edges) that had a mean size outside of the set range were "falsely" discarded. I considered this an acceptable compromise considering the benefit of automatic processing of >4000 sections for more objective quantification of my results. In a typical experiment (Fig 4.2-4.8), I uploaded all images taken from an experimental group usually comprising 5 random field images taken each from the placenta and liver of six individual animals. To try to reduce (my) operator selection bias for a single image, and to gain a better representation of the

overall response in a given tissue, five semi-random, non-overlapping images were generated, and captured using the same microscope settings (Figure 4.2).

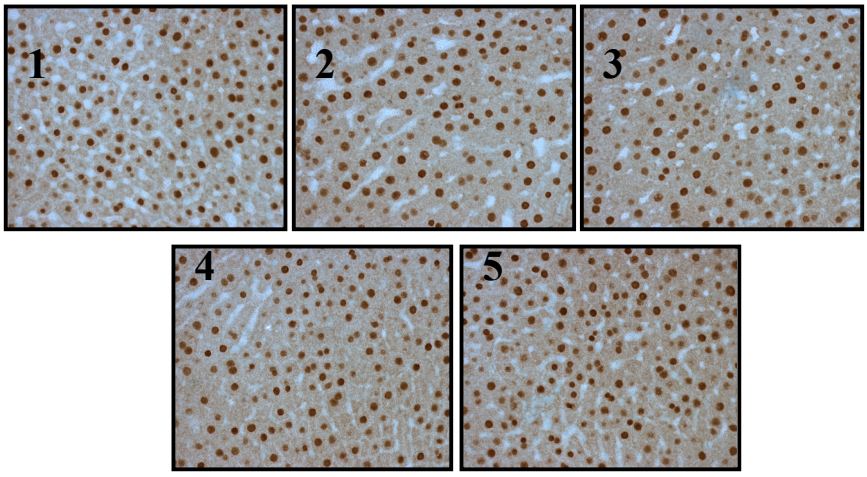
I chose five images as suitable compromise between overload of sections and representation of the section, and found that such semi-random images did appear suitable for practicality to generate quantitative results and sufficient in statistical analysis, and gave results consistent with those obtained by visual inspection. Figure 4.3 illustrates images taken from 5 semi-random fields in liver tissue, observed using an Axioplan microscope and captured with a digital camera on the microscope. The digitised image was then saved as an uncompressed TIFF file that retains all the pixel values of the image. The individual TIFF files were then uploaded as a batch file, into ImageJ. Uploading of the images as batch files increased the speed of the analysis, as well as enabling the same settings to be applied to all images within the batch.

Figure (4.2) Schematic diagram of the method used to generate five semi-random images of liver and placenta section



Slides with stained tissue section were placed on the microscope stage and secured firmly using clips. (X) marks the point of the tissue where it was brought into focus, and the start of the process of generating semi- random images. Each arrow represents a quarter (approx) turn of the stage dial in the direction shown. Each number represents the image field selected for analysis.

Figure (4.3) Images of 5 fields taken from a pregnant dwarf liver



a) Immunohistochemisry for pYStat5 in liver of 16-day pregnant dwarf rat that has received a single injection of bGH, 100µg/100g body weight, 25 min before culling. Images 1-5 are taken from the same animal (as described as above), x40.

The file was then uploaded onto ImageJ and colour threshold set. Figure (4.4) illustrates the display of the example image (a) as seen in the colour threshold display algorithm.

Figure (4.4) Distribution of Hue and Saturation using colour thresholding

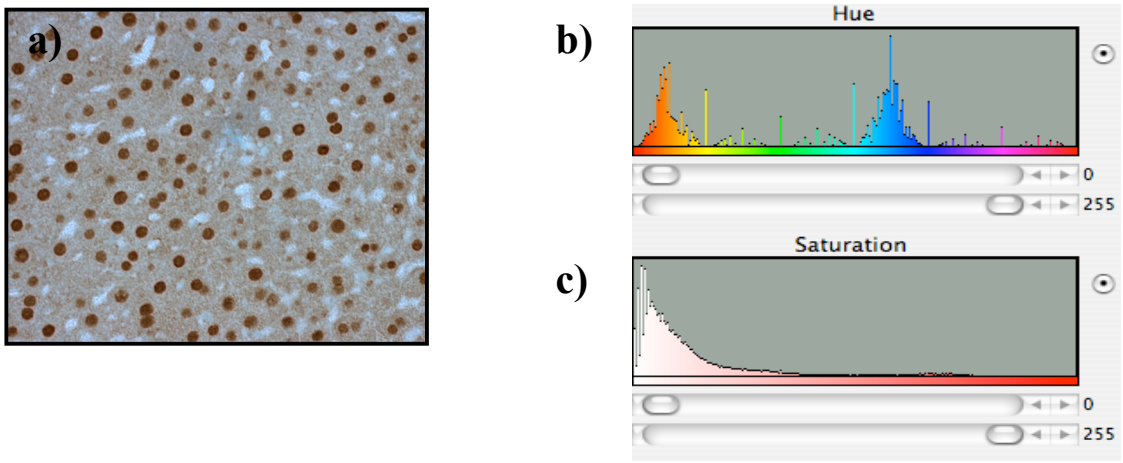


Figure 4.4. a) An example image of 1 of the 5 random fields uploaded as a batch file, b) the distribution of colour (hue) and (c) saturation for this image.

The ImageJ, "colour threshold," parameter was used to display b) the distribution of hue and its value as part of the hue spectrum, and c) the distribution of saturation for the uploaded images. From here I recorded the hue value for "brown" nuclear staining for all the images and then set a mean threshold hue value and range (see later); all images from other experimental groups were then processed with these settings so that objects were identified according to this mean hue value. The background level of colour saturation was determined visually for one of the five random field images and then this value was subtracted from the remaining four. Images were next processed by applying threshold values for object (nuclear) size. The upper and lower range for

non-specific staining size was set the same throughout the quantification of all other images. A working example of the quantification is illustrated in the following figures.

4.2.3 Hue profile for image

The hue profile for the example image using the colour threshold parameter is shown in (Figure 4.5b). Each peak of the hue spectrum corresponds to the mean number of pixels observed for the different parts of the hue spectrum in the example image. Using the hue spectrum to analyze colour, I was able to "gate" in the spectrum, where the brown nuclei are found. In this example the dark brown nuclear staining was distributed within the 1-30 hue range of the spectrum, which corresponds to colours ranging from brown to dark orange and red. There were also a number of other peaks observed in other parts of the hue spectrum, predominately due to the counterstain used in the immunostaining procedure. The counterstain in this case was methyl green (pH 4.6), and the distribution of colour observed was recorded in the 180-200 range, which corresponds to blue and green. The hue range was set to identify positive cells (brown) only, and ignore objects (pixels) with e.g. the 180-200 part of the colour distribution.

In practice, the "brown" nuclear staining was set at 0-25, as 25 was the hue value obtained following the processing of all sections taken from animals given the maximum dose of bGH. This hue range was then used to process all experimental groups including the saline controls. Figure 4.5 shows the processing to objects identified using these hue settings (where the program displays only the objects of the selected "hue").

Figure (4.5) Brown positive cells identified using the hue range 0-25

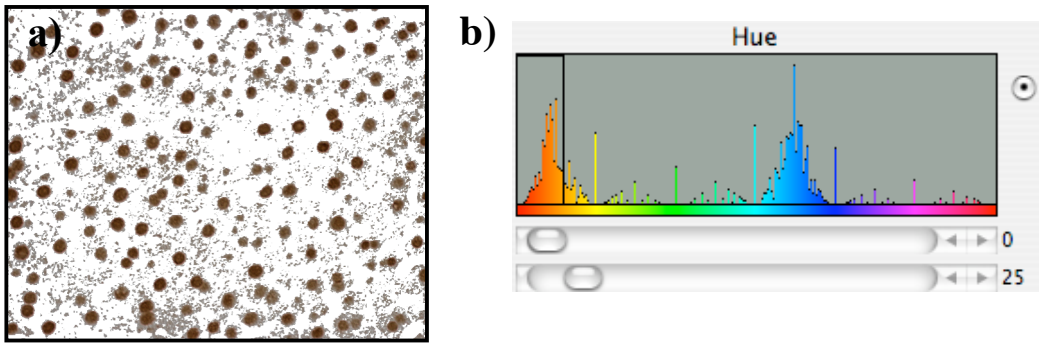


Figure 4.5. a) shows image after the selected hue range is applied. b) Shows the distribution of colour from the original image, shown in fig 5 and the selected range of the hue spectrum, 0-25 (black lines).

By selecting this hue colour range, and checking by visual comparison with the original image, these settings were able to identify the vast majority of responded brown nuclei, identified by visual analysis. However, some fainter stained nuclei were not included if they fell out of this range, so I recognised that this method will moderately under estimate the response. However, I felt this would be similar across different sections, and thus not bias my results.

4.2.4 Saturation profile for image

Like hue, saturation is a measurement that relates to colour but refers to the intensity of a given colour, or, more simply, 'brightness'. Figure 4.6 illustrates how saturation was measured, and then corrected for the example image. The objective of this was to remove background saturation signal that contributed to the intensity of overall staining, whilst discriminating positive (brown) nuclei. Again there are disadvantages to this step. Although most positive cells within the hue, gave a signal above background and saturation threshold ranges set, there were a small number of positive

cells that were fainter in their staining, presenting similar values to background. For this automated process to work effectively, I had to accept that some faint responding cells were likely to have been discarded as background, again underestimating the response. Although all the immunohistochemistry was conducted under the same condition, I still found that the level of background staining varied amongst the sections. For this reason I needed to modify the saturation parameters for each batch analysis, to take into account any differences. However, once set I allowed no variation of saturation amongst the 5 random field images that made up a batch file.

Figure (4.6) Image of liver section, reduction of background noise

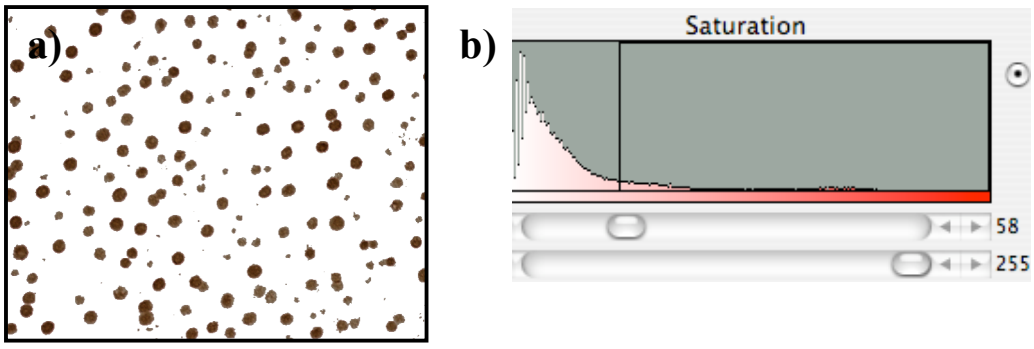


Figure (4.6) a) example liver image with background reduced using the saturation tool bar, the saturation is set to ignore brightness of colour outside of the 58-255 range, as shown in b).

The hue and saturation range set using colour threshold parameters, as well as the upper and lower threshold for nuclei size, which varied very little in full section, were then entered into a final panel, Figure (4.7). All the remaining images of the given batch were then processed with the same settings.

Figure (4.7) Final panel displaying values used in the processing of a batch of five images

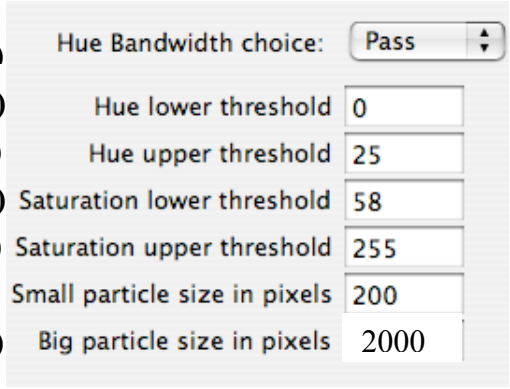
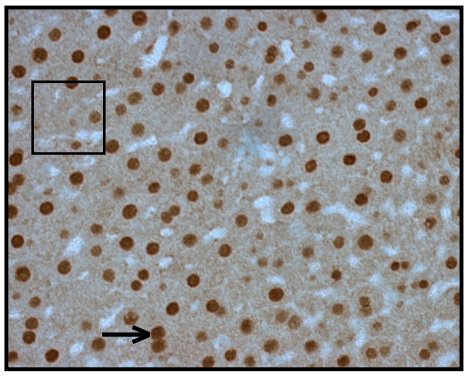


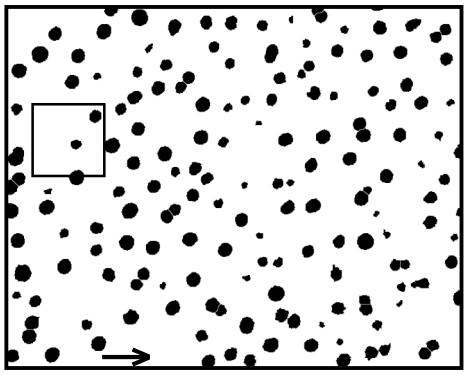
Figure (4.7) a) Hue Bandwidth choice: pass, a setting that refers to the choice to run a set hue range, rather then analysing the image using the whole hue spectrum. Objects were identified in the hue range of 0-25. b) The lowest end of the hue spectrum used to analyse was zero c) the highest end of the hue spectrum used to analyse images, d) Saturation lower range, set as 58. e) Saturation upper range, set at 255. f) Particles less then 200 pixels were discarded and not analysed by the cell analyser. g) Particles over 2000 pixels were discarded.

The results obtained from the quantification were then displayed as a number of images for each random field image, Fig (4.8) and also as a batch raw data file, table (4.2).

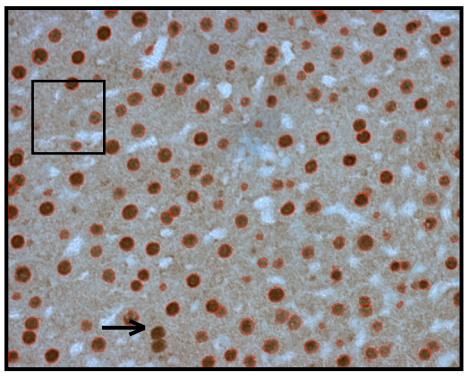
Figure (4.8) an example set of three quantified images produced for each image that made up the batch file



a) Original example image of liver taken from a pregnant dwarf, before quantification is conducted.



b) Mask image, refers to original image after background is subtracted using the saturation upper and lower values.



c) edger image refers to the original image with identified and measured nuclei outlined in red, identified using all the parameters and settings described.

Note: Arrows indicate responded positive nuclei that have not been counted during the automated quantification process; this may be due to the hue of the nuclei not falling within the set hue range or the size of the nuclei being larger then the set upper threshold for object size. The boxes within images indicate areas within the section that the quantification process has discarded smaller and fainter stained objects. Cells may have been discarded as background or because of their size being smaller then the lower threshold set for object size.

Table (4.2) Summary of the calculated raw data for five random field images processed as a batch file for one animal

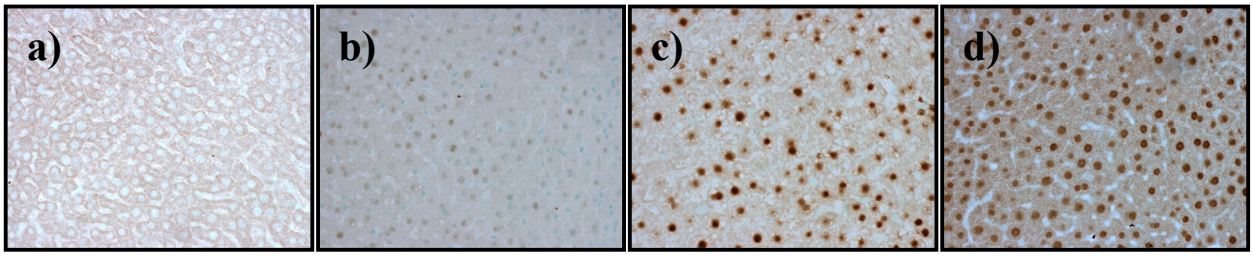
NUMBER OF ANIMAL	NUMBER OF RANDOM FIELD	NUMBER OF BROWN NUCLEI	PERCENTAGE (PIXELS)	AVERAGE HUE	AVERAGE SIZE OF NUCLEI (PIXELS)	AVERAGE SATURATION	AVERAGE INTENSITY
1	1	168	0.12	17	121	971	121
	2	193	0.13	17	125	877	125
	3	188	0.12	17	129	832	129
	4	178	0.16	17	125	957	125
	5	169	0.13	16	126	928	125
Mean	**-**	**179**	**0.13**	**17**	**125**	**913**	**125**

Finally, a mean value was taken for each average of each section for one animal, for these parameters. In the following chapter's results are presented for the response to GH observed in the GHD rat and mouse models using the automated quantification process described above.

4.3 Response to GH follows a dose response pattern in both dwarf rat liver and placenta.

Having established this method, I tested whether the response to GH seen in both liver and placenta was reproducible and whether lower doses would initiate a detectable cellular response to GH in these tissues. Figure 4.9 shows example single images for phosphoStat5 staining generated from groups of 6 pregnant dwarf rats given three different doses of bGH or saline as single intravenous tail injections.

Figure (4.9) PhosphoStat5 Immunostaining for liver sections from dwarf rats treated with varying bGH doses, or saline

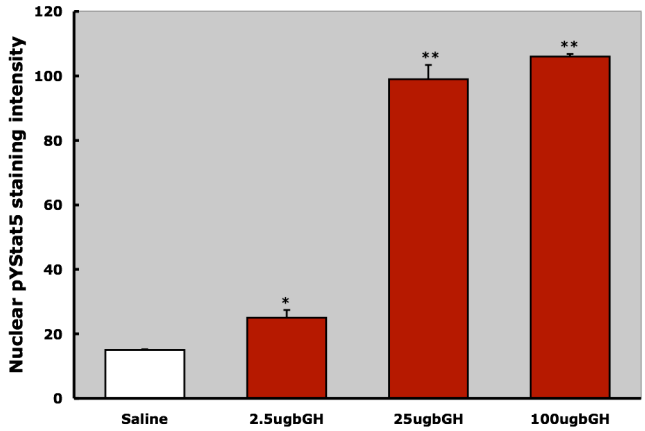


Nuclear pYStat5 staining in liver tissue from pregnant dwarf rats treated with a single injection of (a) saline, (b) 2.5µg bGH, (c) 25µg bGH and (d) 100µg bGH/100g bodyweight. All images are shown at x40, (n=6) for each group.

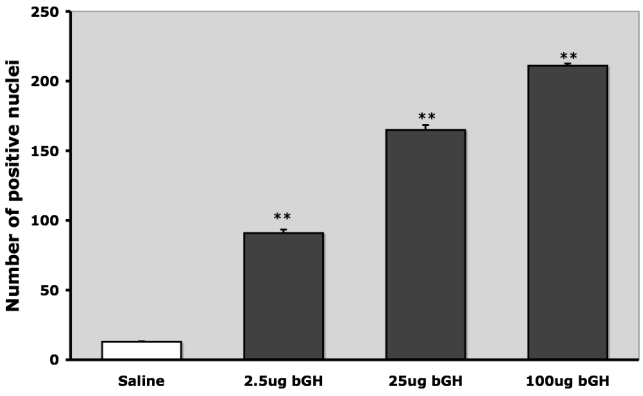
It is clear from Figure 4.9, panels b-d, that the number of nuclei showing staining for phosphoStat5 appear to increase with bGH dose, whereas animals injected with saline only, showed no evident Stat5b staining, suggesting in the absence of endogenous GH, other signals inducing Stat5b in the liver (e.g. Interferon) did not elicit significant signalling. Figure 4.10a and b summarises the level of staining intensity and the number of positive cells calculated for all the images generated from the liver of these animals in this experiment.

Figure (4.10) a) Nuclear staining intensity for pYStat5 b) number of positive (pYStat5) nuclei for liver from pregnant dwarf rats treated with varying bGH doses, or saline

a)



b)



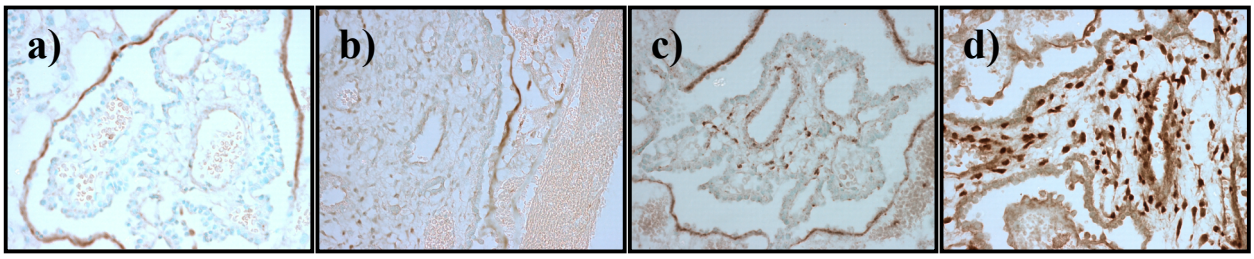
Saline vs varying bGH dose, ANOVA followed with Dunnett's test, *P<0.05, **P<0.01

Figure 4.10a confirms that the liver from pregnant dwarf rats was responsive to bGH in a dose dependent manner, and that there was a small but significant difference in the mean staining intensity between the lowest dose tested (2.5μg bGH/100g bodyweight) of bGH (25.0±2.4) compared to the saline controls (15.1±0.2; $P<0.05$). This dose of GH was therefore able to produce a cellular response in the liver; it is possible that lower doses would also be effective, but it may prove difficult to distinguish this from background, though it is more clear for cell number than for staining intensity. The mean nuclear staining intensity increased with bGH dose; 25μg (99.0±4.4) and 100μg bGH (106.2±0.8) both gave significantly higher responses than the saline control injection (15.1±0.2; $P<0.01$ for both cases). The highest two doses of bGH had similar mean nuclear staining intensities, suggesting that this cellular signal had reached a plateau around 25μg bGH, and that higher dose of bGH would not cause any further response in my system. This does not necessarily mean it has reached maximum effectiveness, but would not exceed the maximum signal in my assay of Stat5b. Figure 4.10b shows a significant dose dependent increase in the number of positive pYStat5 cells, compared to saline injected animals, ($P<0.01$).

4.3.1 The placenta also shows a direct, dose dependent pYStat5 response to GH

The placenta was also immuno stained for phosphoStat5 in the same experimental groups. Figure 4.11 illustrates individual images for the placenta from the same dose response experiments, and again the results from all animals are summarized in Fig. 4.12.

Figure (4.11) PhosphoStat5 Immunostaining for placenta sections from dwarf rat treated with varying bGH doses, or saline



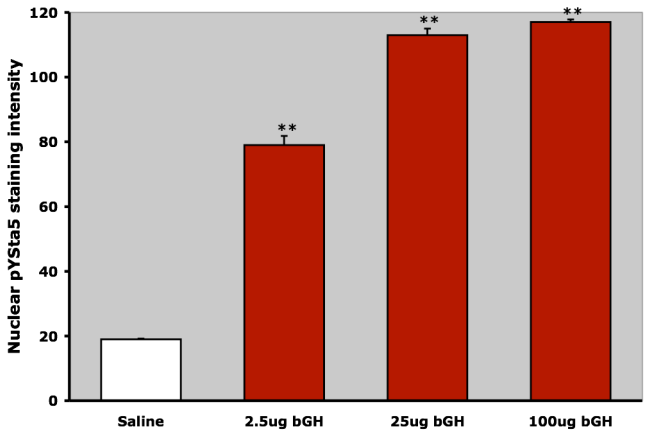
Nuclear pYStat5 staining in placental tissue from pregnant dwarf rats treated with a single injection of (a) saline, (b) 2.5µg bGH, (c) 25µg bGH and (d) 100µg bGH/100g bodyweight. Example images shown at x40, (n-6) for each group.

Strikingly, there was clearly a Stat5b response to bGH in the placenta. Again, a dose dependent increase in the level of staining intensity and number of cell responded was observed, similar to the situation described above in the liver. This confirms my ploted data, showing the placenta is clearly responsive to GH. Figure 4.12a shows that in the placenta there was a dose dependant increase in mean nuclear staining intensity, with a plateauing at the highest dose similar to the liver. All bGH injected groups were significantly higher than the saline group ($P<0.01$). This also shows that the mean nuclear staining intensity recorded for the placenta appeared higher than that of the liver. Figure 4.12b shows the mean number of positively stained cells in the placenta. A significant increase in the mean number of positive cells with increasing bGH dose was shown, when compared to saline controls ($P<0.01$). The highest number of positively stained cells recorded for the maximum dose in the placenta (102) was not as high as in the liver (211). This most likely explained in that staining in the placenta was obviously restricted to specific areas, and as such the staining was

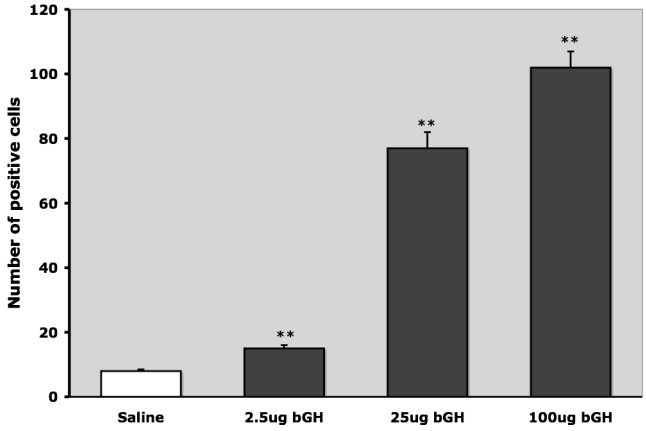
not as homogenously distributed as in the staining in the liver. This makes direct comparison difficult, but it seems reasonable to conclude that the placenta is indeed a direct target of GH action, with strong response of Stat5 phosphorylation in regions of placental tissue in rats.

Figure (4.12) a) Nuclear staining intensity for pYStat5 b) number of positive (pYStat5) nuclei for placenta from pregnant dwarf rats treated with varying bGH doses, or saline

a)



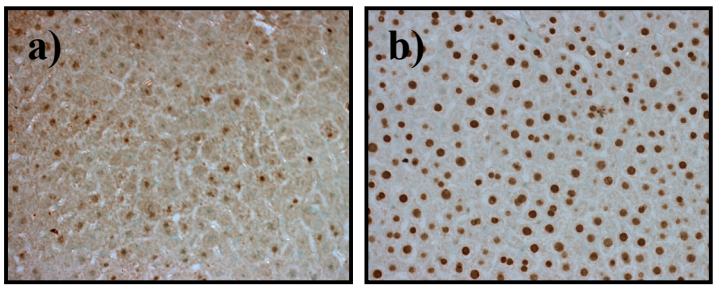
b)



Saline vs varying bGH dose, ANOVA followed by Dunnett's test, **P<0.01

These experiments were carried out in GH-deficient pregnant rats, for technical reasons explained previously. However, I felt it important to see if this could also be shown in non-pregnant animals. Accordingly, I checked the pYStat5 response in the liver of non-pregnant dwarf rats, which received a single injection of bGH or saline (Figures 4.13, and 4.14a & b). Figure 14a clearly illustrates that in this case there are some positive cells in the tissue example for saline injected animals, nevertheless it appears that the number of positive cells, as well as the intensity of staining is higher in the pregnant dwarf animals that have been injected with bGH, (Fig, 4.14).

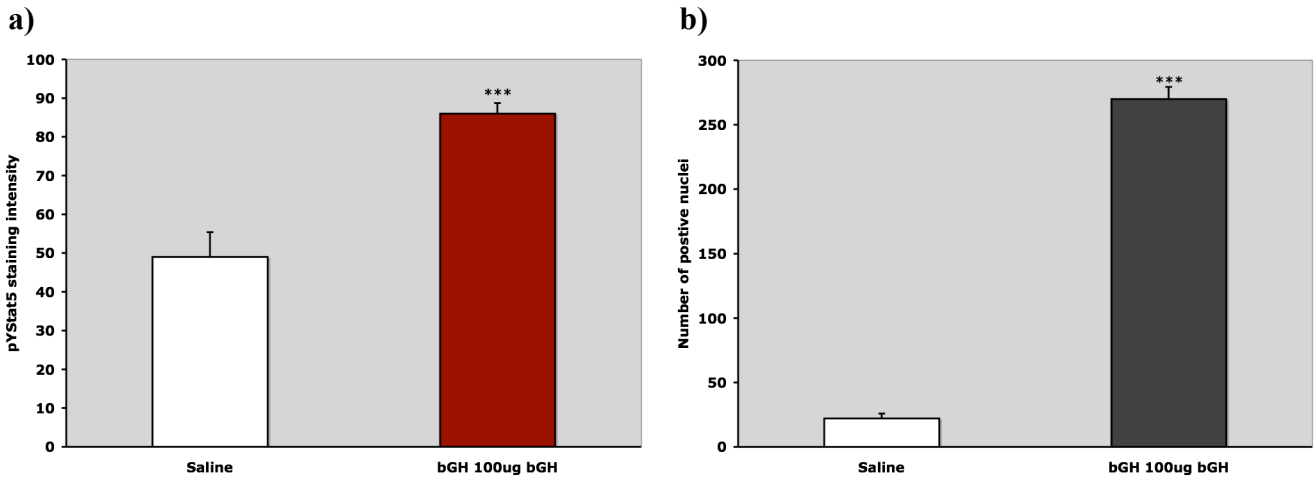
Figure (4.13) PhosphoStat5 Immunostaining in liver sections from non-pregnant dwarf rat treated with saline or bGH



Nuclear pYStat5 staining in liver tissue from non-pregnant dwarf rats treated with a single injection of (a) saline, (b) 100µg bGH/ 100g bodyweight.

Example images shown at x4o, (n=6).

Figure (4.14) a) Nuclear staining intensity for pYStat5 and b) number of positive (pYStat5) nuclei, in liver sections from non-pregnant dwarf rats treated with saline or bGH



Saline vs bGH (100µ/100g bodyweight) injected animals, ***P<0.001 Student t-test.

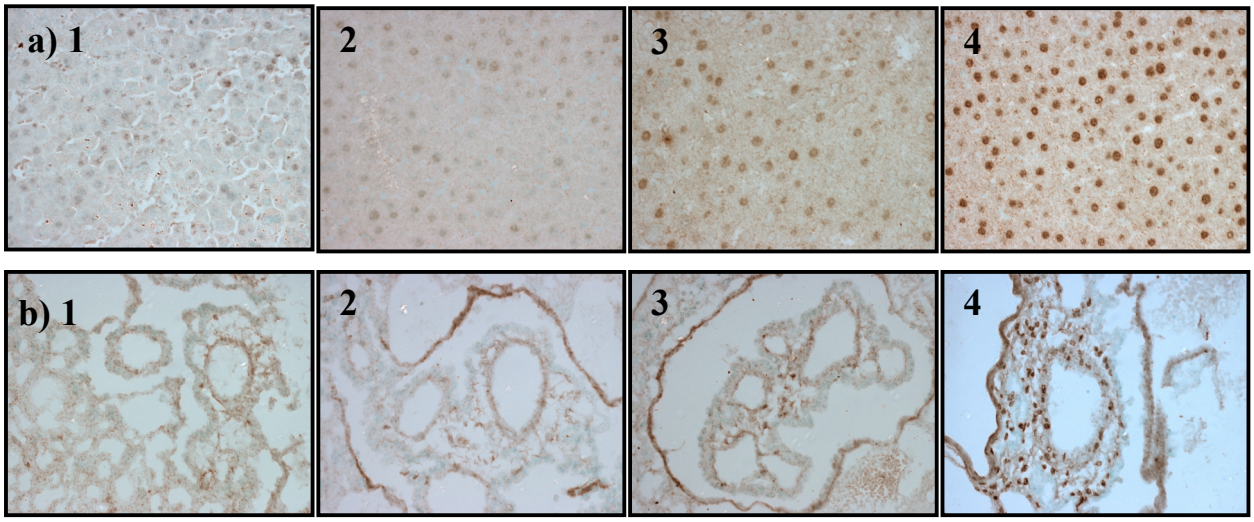
As summarized in Figure 4.14a, the mean nuclear staining intensity in the livers of non-pregnant dwarf rats injected with bGH (86.1±2.7) was significantly higher than that of saline group (48.7±4.5, P<0.001). This indicated that (i) I had a working positive control in non-pregnant GH-deficient rats, and (ii) that the observed response confirmed what has previously been observed by my colleague E.Gevers, in male dwarf rats [291]. Although it is risky to draw direct comparisons between experiments stained on different occasions, it appears that the liver of non-pregnant dwarf rats had a somewhat lower mean nuclear staining intensity compared to that of pregnant dwarfs injected with the same dose of bGH (86.1±2.7 vs 106.2±0.8, respectively), consistent with the idea that the liver becomes more responsive to GH during pregnancy (at least in the absence of circulating GH, that would rise in rats normally). Figure 4.14b shows that the mean number of positively-stained cells in the liver of

non-pregnant dwarf rats given an injection of bGH (270.3±9.4) was significantly higher than rats injected with saline (21.7±3.9, $P<0.001$). Furthermore, the number of positive cells in livers of pregnant dwarfs was lower (211) than the number of positively stained cells in non-pregnant dwarf rats (270).

4.3.2 GH response in normal (AS) pregnant rats

It was also important to test whether GH responses could be seen in tissues in normal (AS) pregnant rats. Figure 4.15 shows liver and placenta sections stained for phosphoStat5 for AS pregnant rats. In both the liver and placenta a dose dependant response to bGH was again seen, as in pregnant dwarf rats. Note that there was a greater number of positive cells after the saline injections in the AS rats than had been seen in the dwarf rats, this is shown particularly in the example liver section (a1), and evident although not as clearly, in the image of the placenta in rats treated with saline (b1). The few positive cells observed in the liver of saline treated AS rats is not unexpected given the presence of endogenous GH pulses seen in normal rats. Nevertheless, it is encouraging that although this method has been developed for analysis mainly in the GH-deficient model a positive response to exogenous GH can still be confirmed in the liver of normal i.e. GH-intact rats.

Figure (4.15) PhosphoStat5 Immunostaining for liver and placental sections from pregnant normal (AS) rats treated with varying bGH doses, or saline



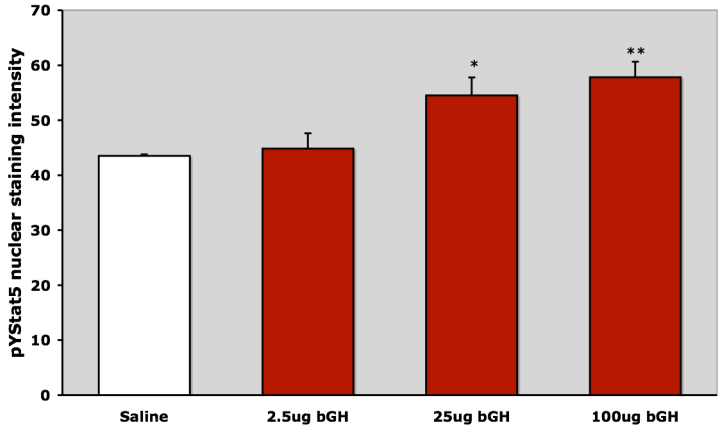
Nuclear pYStat5 staining in liver (panel a) and placenta (panel b) from pregnant wild-type rats treated with a single injection of (1) saline, (2) 2.5µg bGH, (3) 25µ bGH and (4) 100µg bGH/100g bodyweight. Example images shown at x40, (n=6).

Figure 4.16a shows the mean nuclear staining intensity in liver sections from pregnant AS rats. There was no significant difference between the saline injected group and the group injected with the lowest dose of bGH. There was a small but significant increase, however, in the mean nuclear staining intensity for the higher doses of injected bGH over saline controls (54.5±3.3 for 25µg bGH/100g bodyweight and 57.8±2.8 for 100µg bGH/100g bodyweight, compared to saline, 44.83±0.03; $P<0.05$ and $P<0.01$, respectively). Figure 4.16b also showed no significant difference between the saline injected group and the group injected with the lowest dose of bGH for mean number of positive cells counted in the liver. However, there was a

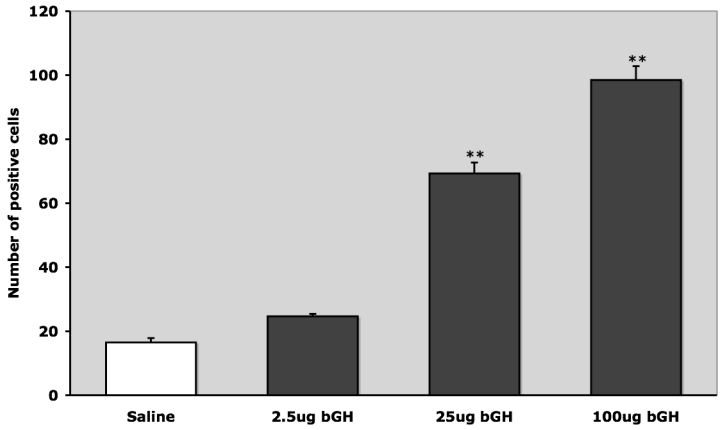
significant dose-dependant increase in the mean number of positive cells between the two higher doses of bGH and the saline group (P<0.01 for both).

Figure (4.16) a) Nuclear staining intensity for pYStat5 b) number of positive (pYStat5) nuclei for liver from pregnant AS rats treated with varying bGH doses, or saline

a)



b)



Saline vs varying bGH dose, ANOVA followed by Dunnett's test, *P<0.05, **P<0.01.

There was no significant difference in either the mean nuclear staining intensity or number of positive cells between the saline-injected animals and those given the lowest bGH dose (2.5μg/100g of bodyweight). This contrasts with the previous results in dwarf rats, in which the same dose of GH in pregnant dwarves induced a significantly higher response than hepatic Stat5 seen in the saline group. An obvious explanation for this difference in dose sensitivity would be that the level of endogenous GH present in the AS rat causes some basal signalling and reduces sensitivity to a further GH challenge. Therefore even after an injection of saline I expected to see some level of pre existing cellular response (due to endogenous GH), and a shallower dose response curve. Later experiments in this thesis provided some support for this notion. Finally, it was also shown in both a) and b) of Figure 4.16 that the highest two doses of bGH, generated lower mean nuclear staining intensity and fewer positive cells than I saw in pregnant dwarf rats. Thus I conclude that the response to a single injection of bGH in pregnant AS rat liver is not as strong in pregnant dwarf rat liver. Again, I suspect this is most likely to be desensitization due to the endogenous GH present in normal rats, as also reported previously by Gevers *et al* (2009) [282].

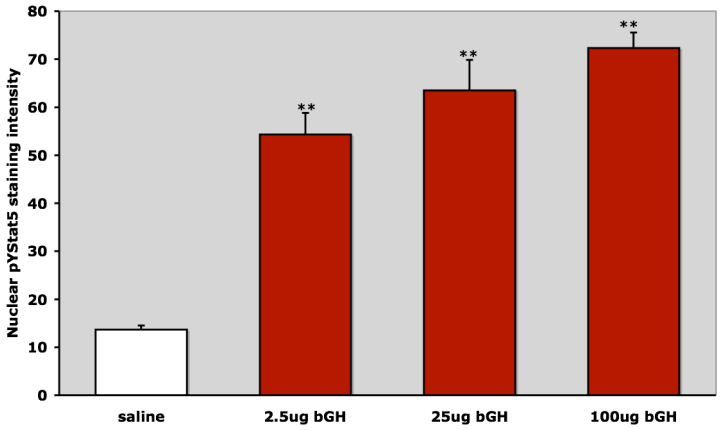
4.3.3 GH responses in the placenta of normal AS rats

Figure 4.17a and 4.17b show that the placenta from AS rats also showed a dose-dependent response to GH, though the dose response for staining was quite shallow. Despite this, I found a significant increase in mean nuclear staining intensity for all doses of bGH compared with saline injections, ($P<0.01$). There was also a similar dose dependent increase in the mean number of positively stained cells, compared to saline controls ($P<0.01$), with a more pronounced dose-dependent relationship.

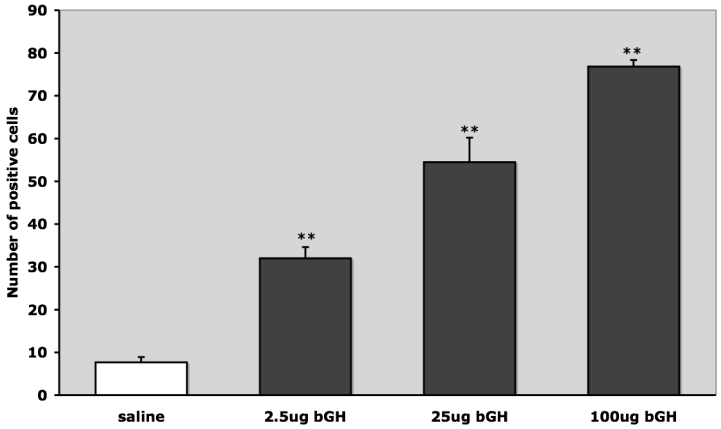
Clearly therefore, the normal (i.e. non-GHD) placenta responds to GH with a pYStat5 response. Thus if anything the evident response might suggest that the placenta is less susceptible to the desensitization response of endogenous pituitary GH, which could be interesting if true, as it may be more continuously exposed to a paracrine source in humans at least.

Figure (4.17) a) Nuclear staining intensity for pYStat5 b) number of positive (pYStat5) nuclei for placenta of normal rats treated with varying bGH doses, or saline

a)



b)

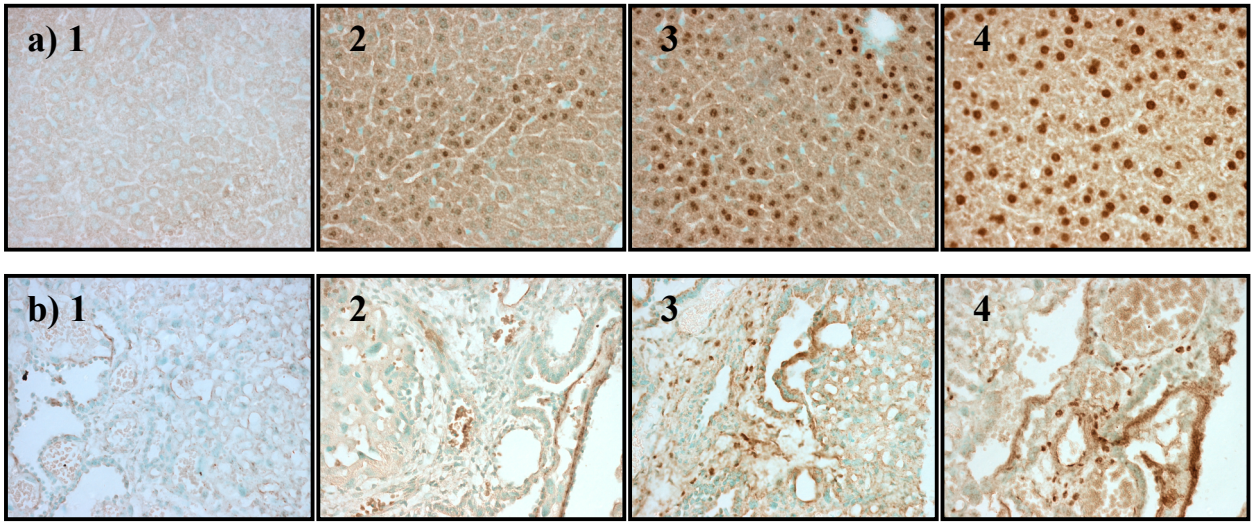


Saline vs varying bGH dose, **P<0.01. ANOVA followed by Dunnett's test.

4.4 Visualizing the response to GH in the liver and placenta of growth hormone deficient (GRF-M2) and wild-type mice

I felt it was important to see if I could obtain similar results in at least one other GH-deficient rodent model, to exclude it simply being a property specific to the dwarf rat. Similar experiments were therefore repeated in GH-deficient transgenic (GRF-M2) mice, and compared to non-transgenic (wild-type) mice. Figure 4.18 shows immunoassaying for phosphoStat5 in mouse liver and placenta. As before, individual images are shown first followed by figures summarizing the group data in the next few figures. The data essentially confirm the results I obtained in dwarf rats.

Figure (4.18) PhosphoStat5 Immunostaining for liver and placental sections from pregnant GRF-M2 mice treated with varying bGH dose or saline



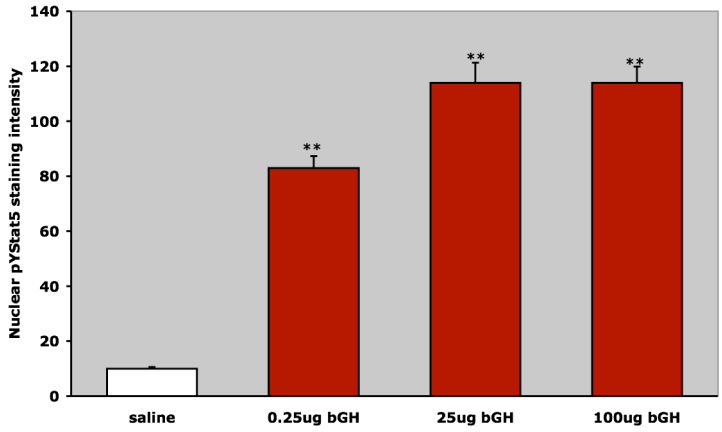
Nuclear pYStat5 staining in liver (panel a) and placenta (panel b) from pregnant wild-type mice treated with a single injection of (1) saline, (2) 0.25µg bGH, (3) 2.5µg bGH and (4) 50µg bGH/100g bodyweight. Example images are shown at x40, (n=6).

I observed a response in the mouse liver to bGH and, importantly, confirmed Stat5b response to GH in the mouse placenta. Again, this is the first time this has been reported in this tissue in this species.

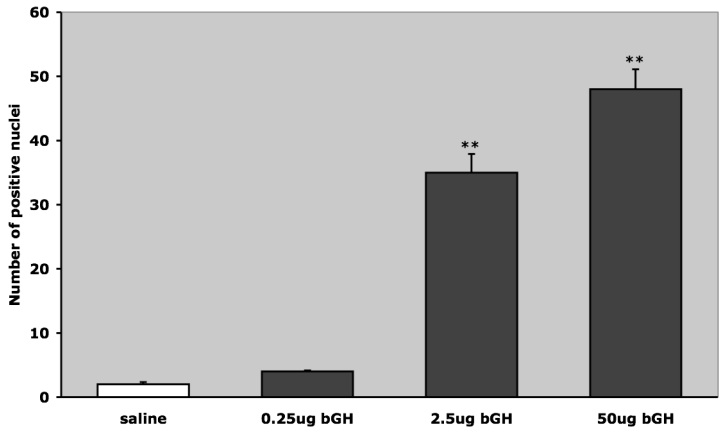
Figure 4.19a shows that there was a significant increase to all doses of bGH in mean nuclear staining intensity in the liver compared to saline injections ($P<0.001$). Figure 4.19b shows no significant difference in number of positive cells in the saline group and the lowest bGH dose (0.25μg bGH/100g bodyweight), but a significant difference between the saline controls and higher doses of bGH (though the dose response relationship at these doses for staining (a) was quite shallow). The result for the low GH dose were interesting, with a significant increase in staining intensity, but not number of responding cells. One suggestion is a differential dose effect revealed in these mice experiments, initially increasing staining in already responsive cells, subsequently recruiting more responsive cells (Fig 4.19 a vs b), as the GH doses is increased, but without increasing the intensity of the response further. It is also possible the responses are more “all-or-none” in the mouse.

Figure (4.19) a) Nuclear staining intensity for pYStat5 b) number of positive (pYStat5) nuclei from liver of GRF-M2 mice injected with varying bGH doses, or saline

a)



b)



Saline vs varying bGH dose, ANOVA followed by Dunnett's test, **P<0.01.

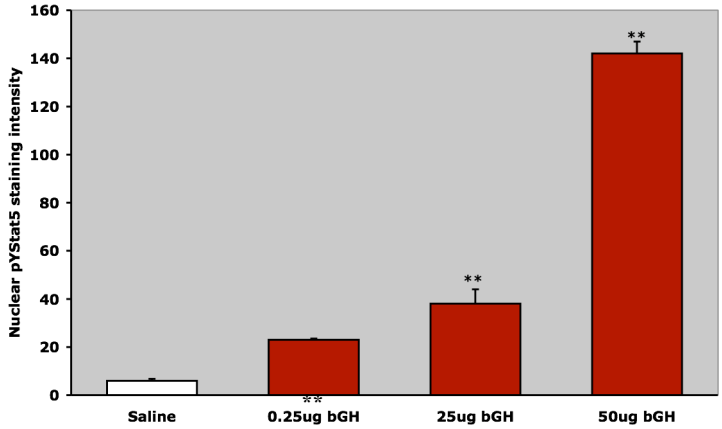
In the same experiments, Stat5b response to a single injection of bGH was clearly observed in the placenta. Figure 4.20a suggest that the initial dose of 0.25µg bGH initiated a significant response in the placenta when compared to the saline group (20.0±0.5, compared to 10.0±0.8, P<0.01). Figure 4.20b, also shows that the group receiving, the lowest bGH dose showed a higher number of positive cells compared with the saline injected group (1.7±0.2 vs 3.7±0.2, P<0.01). Moreover, the higher

doses showed highly significant dose dependent trend for both mean nuclear staining intensity (39.5±.02 and 142.1±5.7) and the number of positive cells (61.5±3.8 and 124.0±3.2), for 2.5μg and 50μg, respectively. This clearly establishes the mouse placenta as a direct target for GH action.

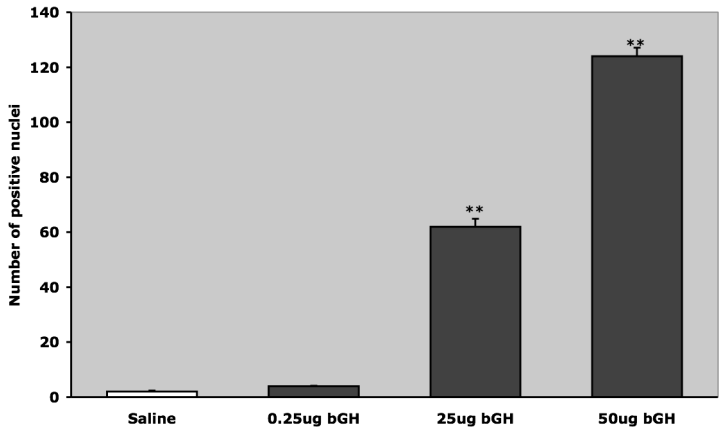
Interestingly, there appeared to be higher nuclear staining intensity in the livers of GRF-M2 mice compared to dwarf rat liver. This might have been due to the mouse model being functionally more GH - deficient than the dwarf rat model, therefore the response shown to a GH injection would be more intense with the reduction of background (endogenous GH) interference. However, the degree of pituitary deficiency with dwarfs is similar, so this is unlikely. Other reasons for the difference in species may include the difference of the amount of Stat5 or the density of cells in the liver of both models. Also, one cannot rule out technical differences that may have arisen during the handling and processing of tissue in different experiments rendering direct comparisons risky. The results obtained for nuclear staining in the placenta showed a higher number of positive cells in rat placenta, compared to mouse. Again, it is quite probable that many differences in both species, including size of rodent and placenta may contribute to the degree of responses to GH. Overall however the important point is that both rodents show placental Stat5b phosphorylation response to a single GH injection, which is the hallmark of a GH-responsive tissue.

Figure (4.20) a) Nuclear staining intensity for pYStat5 b) number of positive (pYStat5) nuclei for placenta from GRF-M2 mice injected with varying bGH doses, or saline

a)



b)



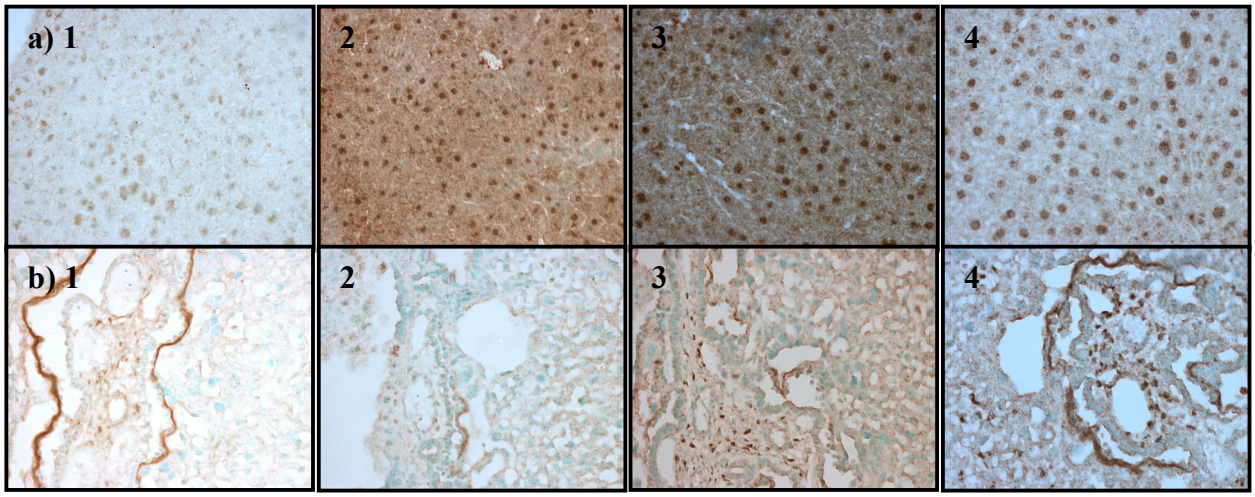
Saline vs varying bGH dose, ANOVA followed by Dunnett's test, **P<0.01.

4.4.1 Pregnant non-transgenic mice have a similar dose dependant response to GH as transgenic model

As before, I wished to test whether these responses could also be seen in pregnant wild-type mice (with an intact pituitary GH axis). Accordingly, figure 4.21 illustrates immunostaining for phosphoStat5 from wild-type mice given saline and varying bGH doses (1-4) in liver panel (a) and in placenta (b). Both tissues showed dose dependent effects in the level of staining intensity and the number of positive cells recorded in

response to GH. A low basal signalling could be detected in the saline-injected group, but the responses were faint.

Figure (4.21) PhosphoStat5 Immunostaining for liver (panel a) and placental sections (panel b) from pregnant non-transgenic, wild-type mice treated with varying bGH doses, or saline



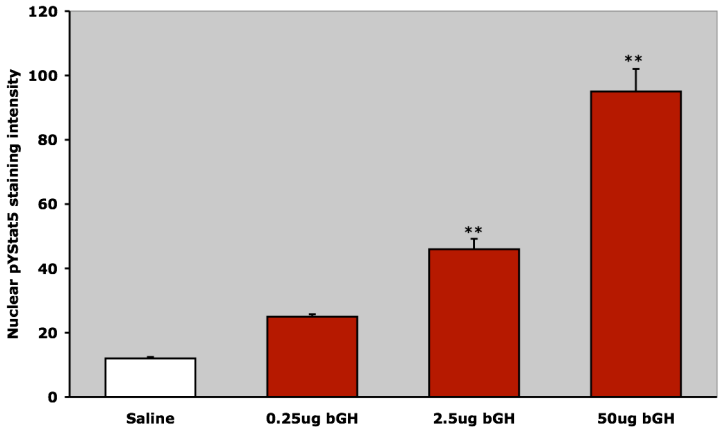
Nuclear pYStat5 staining in liver (panel a) and placenta (panel b) from pregnant non-transgenic wild-type mice treated with a single injection of (1) saline, (2) 0.25µg bGH, (3) 2.5µ bGH and (4) 50µg bGH/100g bodyweight. Example images were created using x40, (n=6).

The hepatic responses for all the animals are summarized in fig, 4.22. There was no significant difference in mean nuclear staining intensity (Fig 4.22a) in the livers of animals injected with saline and those injected with the lowest dose of bGH (0.25µg/100g bodyweight), 11.7±0.4 compared to 24.0±0.7. This indicates that, as in wild-type rats, in wild-type mice the level of response measured in the lowest dose of bGH is difficult to distinguish from response to prevailing endogenous GH levels. However, there was a significant increase in mean nuclear staining intensities for further increasing doses of bGH compared to saline, (45.8±3.2 and 94.5±7.1 compared to 11.7±0.4, $P<0.01$). Figure 4.22b shows a similar pattern in mean number

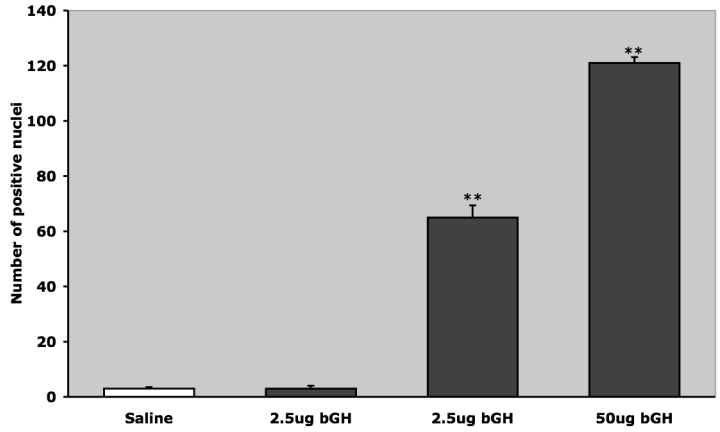
of positive cells. There was no significant difference between the saline and the lowest bGH injected animals (2.8±0.5 vs 5.2±1.0). However, a dose dependent increase was found with the higher doses of GH (64.8±4.4 and 120.5±2.1 vs saline (2.83=±0.5), P<0.01).

Figure (4.22) a) Nuclear staining intensity for pYStat5 b) number of positive (pYStat5) nuclei for liver from non-transgenic, wild-type mice injected with varying bGH doses, or saline

a)



b)



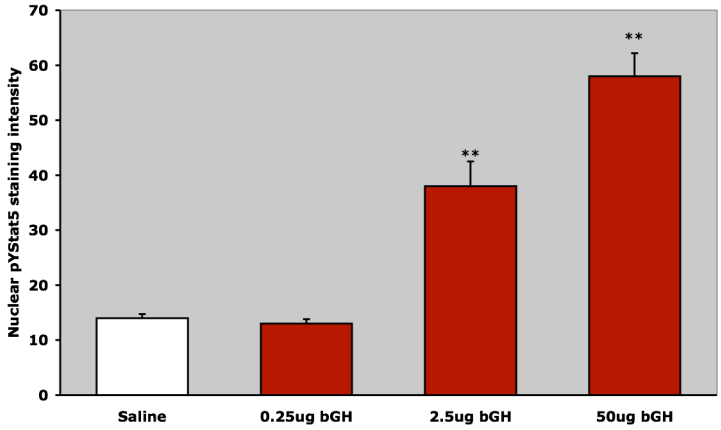
Saline vs varying bGH dose, ANOVA followed by Dunnett's test, **P<0.01.

Figure 4.23 simply summarizes the *placental* responses to GH in these normal mice. Figure 4.23a shows that the placenta had the same dose-dependent responses as the liver. Again, there was no significant difference between the saline and lowest bGH dose for the mean nuclear staining intensity, 14.0±0.7 compared to 13.2±0.8. However, a significant increase in nuclear staining intensity was seen in higher doses of bGH, compared to the saline group (37.0±4.5 and 58.7±4.2 compared to 14.0±0.7, $P<0.01$). There was a significant increase in the mean number of positive cells (Fig 4.23b) with increasing bGH doses compared to saline. Thus, normal mouse placenta was clearly a target for GH, and the response was not confined to dwarf GHD animals, but also evident in GH-normal animals.

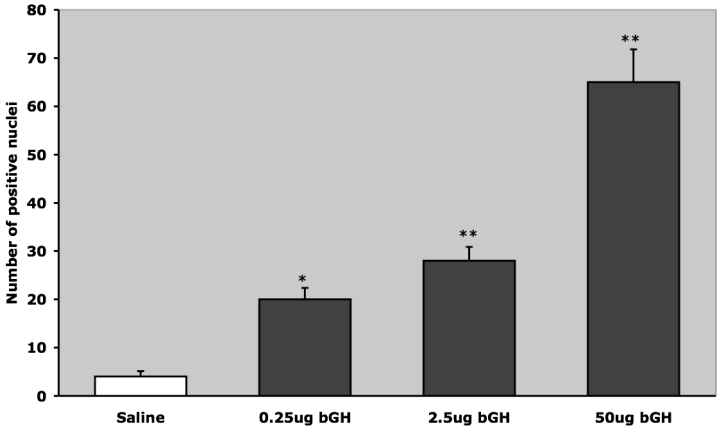
Again I noted that there appeared to be a lower level of GH-induced nuclear staining intensity, and number of positively stained cells, in both the liver and placenta of wild-type mice compared to GRF-M2 mice. This was also seen in pregnant wild-type rats compared to dwarf rats. As for normal rats, I felt the most likely explanation is that wild-type mice have prevailing endogenous GH signaling, which could blunt the response to an acute GH challenge

Figure (4.23) a) Nuclear staining intensity for pYStat5 b) number of positive (pYStat5) nuclei for placenta from non-transgenic, wild-type mice injected with varying bGH doses, or saline

a)



b)



Saline vs varying bGH dose, ANOVA followed by Dunnett's test, *P<0.05 and **P<0.01.

4.5 Discussion

My results from experiments in non-pregnant dwarf rats have reproduced the pYStat5 responses to GH previously described by Gevers *et al* (2009) [291], who originally developed this method, to show a single acute injection of GH, in a GH-deficient animal, through visualization of phosphorylated Stat5. What I have added to the method is an analytical semi-automated process that allows this response to GH to be analyzed using a relatively objective set of quantification parameters, applicable to large numbers of sections, for high throughput, and affording statistical comparisons between groups. As expected non-pregnant dwarf rats clearly showed higher nuclear staining intensity for pYStat5 and an increased number of positive cells in response to GH, compared to animals injected with saline. This adds to previous data mostly obtained in male rats, but my main aim was then to test whether a response to GH, if any, could be detected in the liver in pregnant females, and in particular, whether responses could also be detected in this placentae. I also wished to test this in another GHD rodent species.

My results are the first to show that pregnant dwarf rats given an acute injection of GH, exhibit an increase in both staining intensity, and number of positive cells, compared to saline injected animals, in placenta as well as liver. Importantly, the responses to GH appeared to increase according to GH dose in the liver, and suggests that both the responding tissue and the method of analysis was able to detect changes in staining intensity and the recruitment of responding cells, with increasing GH dose.

The level of nuclear staining intensity observed in pregnant liver appeared higher than that recorded for liver from non-pregnant dwarfs, however, the number of responding cells were appeared the same. This suggests that the same number of GH responsive cells is present, and that additional cells are not recruited in pregnancy. It is unclear why this might be, though it is possible there is more Stat5 present (to be phosphorylated) or a higher Jak kinase activity. An important conclusion however, is that there may be "silent" GH-responsive cells which become "visible" only at higher GH doses.

The same observations were made in AS rats, in which I observed a significant response to GH, compared to rats injected with saline, however, there was a less obvious dose dependant pattern to GH shown, compared to that evident in dwarfs. The obvious explanation I have advanced for this is that endogenous GH is present, which makes it more difficult to record a clear response to an acute injection of GH in the AS rats. This could be by desensitization of the response acutely, as more long-term adaption of the JAK/Stat system in the presence of normal GH. I performed some GH infusion experiments, described in chapter 5, which lends some support to this idea. Further evidence for this is the higher level of background staining in the AS livers, compared to that in dwarfs. There are other ligands that can activate Stat5 (e.g. IFN) [348] so I cannot rule out that some of this staining, may be due to Stat5 signal unrelated to GH. It can also be that washing during the immunohistochemistry wasn't as effective, though this would be the same for all sections in the batch, treated and untreated. Nevertheless, due to the subtraction of background, there will likely have been some loss of true staining, which could have reduced the signal recorded for nuclear staining intensity, though why this should differentially affect AS livers is

unclear. However, it is important to note that the number of positive cells in the AS rats did increase with dose, to similar numbers shown in the dwarf livers. Despite the level of response within AS liver being lower, the number of responding cells per section was similar between dwarf and normal animals.

The main novel finding addressing my main aim in convincing fashion, was that in the same pregnant animals direct GH-induced YStat5 responses were clearly evident in placenta, and that the placenta from both dwarf and AS rats does represent a direct target for GH. It was obvious however that, unlike the liver, responding cells were confined to one given area and structure of the placenta, which appears to be syncytiotrophoblast cells. As with liver, the placentae from dwarfs showed a significant increase in both nuclear staining intensity, and number of positive cell, according to an increase in dose. Similar results were seen in placentae from AS rats, however, both nuclear staining intensity and number of positive cells recorded were lower than for dwarfs placenta. This implies that the placenta from AS rats may be subject to the same difference as seen in responses in the liver from AS rats, i.e. less responsive to an acute injection of GH. Again, it is reasonable to assume that endogenous GH levels in AS rats contribute to this desensitization, also in the placenta.

To show that this was not a unique property of dwarf (GHD) rats, the same experiments were carried out in a transgenic GH-deficient mouse model (GRF-M2). When GH injection experiments were repeated in these mice, liver from both GRF-M2 and non-transgenic mice showed significant increases in nuclear staining intensity, as well as, number of positive cells. Interestingly, the non-transgenic mice, like AS rats, showed lower levels of staining intensity, compared to GRF-M2 mice,

consistent with their endogenous GH being much higher. Furthermore, the number of positive cells recruited with varying dose in the non-transgenic mice, were similar to numbers recorded for GRF-M2 mice, again consistent with my results in GHD rats. This indicates that non-transgenic mice may be similar to AS rats, in that the levels of intensity of response is reduced due to spontaneous GH, however, in this case, this didn't appear to alter the number of cells responding. However, it is also important to note that the high levels of background observed for AS rat liver was not equally observed for wild-type mouse liver, so not all the results can be directly extrapolated between these species.

Gratifyingly, I was able to confirm placental Stat5b responses to GH response in mice. Placenta from both GRF-M2 and non-transgenic mice showed responses in the same syncytiotrophoblast regions, with dose dependent increases in nuclear intensity and number of positive cells recorded. I can therefore conclude that the mouse placenta is also a direct target for GH in the mouse. As in the liver from non-transgenic mice, placenta from non-transgenic mice also showed much lower levels of staining intensity, and number of positive cells, compared to GRF-M2 mice, again implying a contribution of endogenous GH levels to placental responsiveness to an acute action of GH. I noted that the placenta and liver in GRF-M2 mice also showed much higher levels for nuclear staining intensity, and number of positive cells, compared to dwarfs. This differs from normal rodents, which showed similarities in these tissue responsiveness to GH, but I am cautious extrapolating this, as there are differences in the origin, severity, and cellular basis of GH-deficiency in my two models of GHD.

The quantification package I developed proved helpful for analysis of tissue responses in both mice and rats, but some comments are warranted about the difficulties encountered when analyzing the two tissue types. One difference observed for the placenta, compared to the liver, was the higher levels of non-specific staining. The non-specific staining shown in the placenta also had an edge like effect, outlining several structures, which were more troublesome for analysis given the relative heterogeneity of the placenta compared to the rather more homogeneous hepatic sections. This kind of non-specific edge staining was not observed in the liver. I cannot rule out that this type of "background" staining is in fact specific, however, it is not characteristic of the nuclear pYStat5 staining shown in the liver and the other parts of the placenta. Nevertheless, its removal during the analysis generates a potential underestimate of weakly responding cells, that get "subtracted" in the automated analysis, which would underestimate the overall response. Formally, I can thus describe positive cells as "responders", but "negative" cells may be missed as weak responders not "non-responders" as I classify them here.

A second concern is that the cut off points for non-specific staining was set using the mean size of stained nuclei, so that any object size outside of the upper and lower settings would be discarded as non-nuclear and non-specific. However, edge-like nuclear staining in the placenta would lie within these settings, and therefore could have resulted in the creation of false positives. A way of reducing this would have been to set non-specific cut-off settings differently according to the tissue being analyzed, but I felt this risked introducing too much subjectivity into the analysis, I therefore preferred to accept the limitations as they are, and maintain unbiased

comparisons in the analytical settings. Nevertheless, this inhibits me from drawing too firm comparisons quantitatively between tissues, especially with lower doses of GH.

Another difficulty is that some of the nuclear staining observed for the lower doses of GH was very faint, compared to staining achieved from higher doses. Visual inspection of some of these sections suggested that on occasion subtraction of background non-specific staining probably resulted in "missing" these fainter signalling nuclei. In some sections a visible difference between lower GH dose and saline was apparent, however, following the automated processing the same images, for the responses lower GH doses were not significantly different to images from animals injected with saline. To over come this, the background subtraction values could have been changed to compensate for fainter signals, however, this would then introduce the problem of allowing some real non-specific staining to come through and it is always possible the visual impression is misleading. Again, I felt the practical advantage of the higher throughput, semi-objective method, outweighed these errors, which would mostly be a problem at lower doses of GH, and it was better to accept these limitations.

In summary, I have shown that the liver responds to bGH during pregnancy in dwarf rats. Furthermore, the results indicate that this response was much more accentuated during pregnancy compared to the liver of non-pregnant dwarf rats. A response to bGH has been documented for the first time in the placenta, which has been shown in both dwarf and GRF-M2 mice. Both the liver and placenta showed similar response to GH, in a dose dependent manner. A single injection of bGH was less effective in both

the liver and placenta in normal rodents, when compared to GH-deficient rodents, suggesting the latter are more sensitive to GH presented in this way.

Finally, the quantification method devised to analyze GH responses proved to be effective and useful. It may require slight adjustments according to tissue type for more robust quantification of GH responses, but an acceptable compromise between false positive and false negatives allowed quantitative comparisons of GH responses and thus statistical evaluation to be performed on large numbers of images.

5. GH-signaling in pregnant rat liver and placenta: GH secretory pattern and nutrition

5.1 Introduction

Both the pattern of GH secretion [52] and changes in nutrition [349] alters the level of GH response in the liver, as reviewed in the Introduction. Having established a method for quantifying the responses to GH, I then performed experiments with the following aims: (i) to visualize potential differences in the hepatic response to GH given in different patterns in pregnancy, (ii) to look for changes in GH responses in the placenta, and (iii) to examine whether there are differences in response to GH, in the liver or placenta, under nutritional restriction.

5.2 Experimental approach to investigating the effects of different GH patterns

In the previous chapter, I showed that during pregnancy the liver and placenta responds to a single acute injection of GH, with increases in pYStat5. Whilst this single GH pulse is optimal for visualizing and identifying responsive cells, it is clearly not an appropriate physiological GH exposure shown during pregnancy, which is more continuous. To address this, I sought to test phosphorylation of Stat5 in pregnant dwarf rat liver and placenta after a 7-day exposure to GH, delivered by a subcutaneous osmotic pump in a continuous fashion.

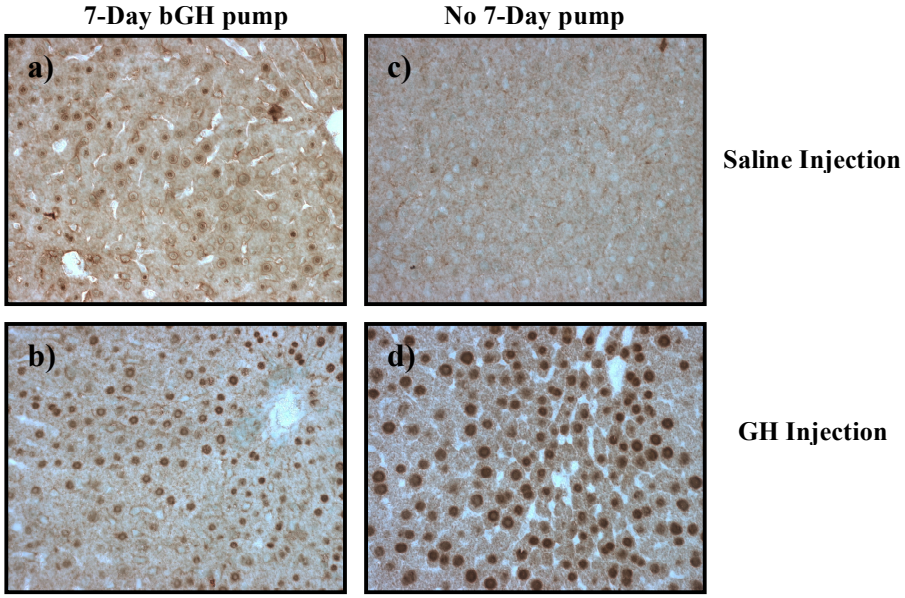
Growth in rats is dependent on the pattern of GH exposure [50]. A study by Gevers *et al* (1996) [78] generated comparative dose-response curves for dwarf rats treated with pulsatile or continuous intravenous infusions of GH. They showed that pulsatile infusions increased weight gain and bone growth, more than continuous infusions, for any given GH dose. The total duration of treatment in these experiments was 7 days, with a maximum dose of 200μg/day. Continuous infusion at this dose continued to

induce increased growth, with no observable plateau reached. In contrast, dose-response curves for dwarf rats treated with pulsatile treatment of GH began to plateau after doses above 72μg/day. In the same study, when mixed patterns of GH pulses superimposed on continuous GH exposure were given to animals, the pulsatile effect was predominant for growth, (though growth was stimulated to some degree by continuous component), I therefore decided to test a 7-day continuous infusion of 200μg/day bGH, which could more likely mimic physiological exposure during pregnancy.

5.2.1 Effects of continuous GH exposure on hepatic pYStat5 responses

Figure 5.1 illustrates images from the livers of pregnant dwarf rats treated with or without continuous bGH for 7 days, followed by an acute injection of saline or bGH, to test the pYStat5 response. Figure 5.1(a) shows an example of liver tissue from a dwarf rat treated with a 7-day continuous infusion of bGH (pump), followed by a single acute injection of saline alone i.e. no acute GH challenge. I observed a number of nuclei with pYStat5 staining and an overall increased staining intensity with continuous GH exposure. Figure 5.1 (b) shows section of liver tissue from an animal treated with a 7-day continuous bGH infusion, but also given a single acute injection of bGH, shortly before sacrifice. In these animals I observed more responding nuclei, which also appeared to have a higher level of staining intensity for pYStat5 when compared to (a). Figure 5.1 (c) shows an example of liver tissue from an animal not pretreated with continuous 7-day bGH infusion, and only given a single acute injection of saline (double control). In this example I observed a low number of pYSta5 nuclei staining, with less staining intensity, compared to (a) and (b). However, the strongest pYStat5 response to GH is shown in the section in (Fig 5.1 [d]), taken from an animal treated with a single acute injection of bGH, without prior continuous GH exposure.

Figure (5.1) Liver sections from dwarf rats, immunostained for Phospho Stat5,+/- 7 day bGH pump, followed by an acute iv injection of saline or bGH



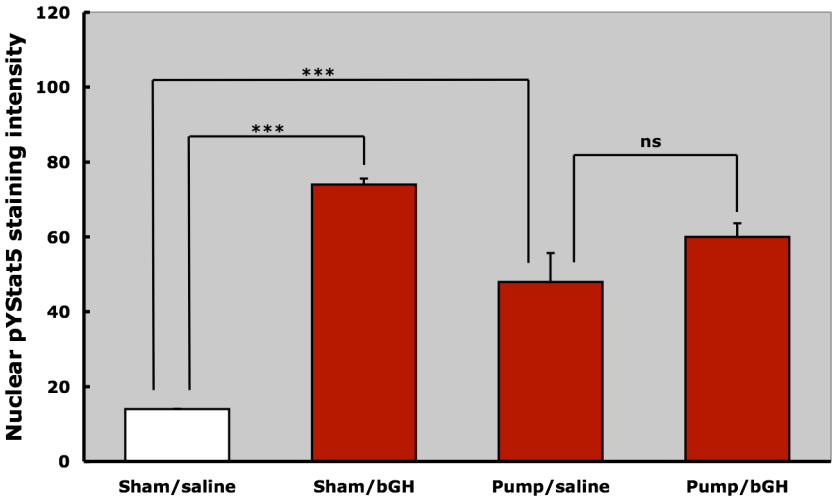
pYStat5 staining in the livers of pregnant dwarf rats treated (a) with continuous bGH infusion (pump) for 7 days and then with an acute injection of saline (b) with bGH. In (c) dwarfs received no pretreatment with continuous bGH, but given a single acute injection of saline, or bGH in (d). (n=6) for each group.

The result of quantification of all the sections from groups of 6 animals with these treatments is shown in Figure 5.2. Figure 5.2a shows that the livers in pregnant dwarf rats were clearly responsive to continuous bGH infusion; nuclear staining intensity for groups treated with continuous bGH infusion was significantly higher (48±7.7) than that of the control group (14±0.1, $P<0.001$), which received no pre-treatment with continuous bGH infusion. Figure 5.2b shows a similar significant increase in the mean number of positive cells in the group that received continuous bGH infusion (35±8.6), compared to the control group (2±0.2), $P<0.05$. Giving an acute injection of bGH on top of continuous bGH treatment produced an apparent further increase in mean

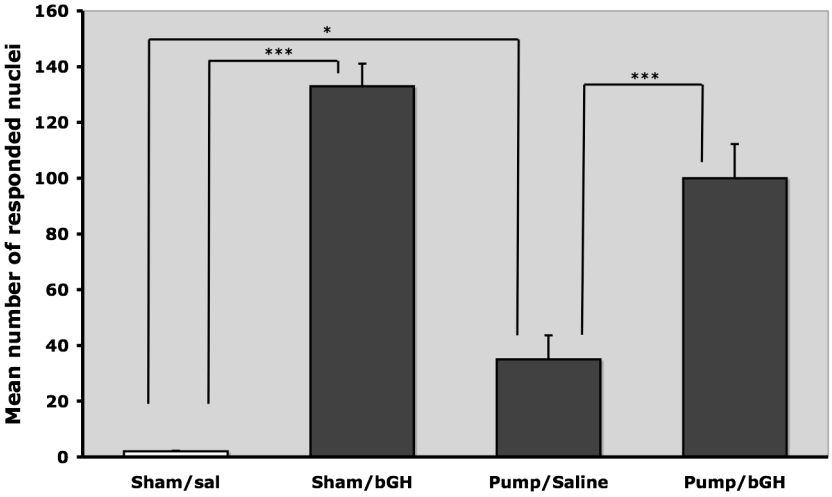
nuclear staining intensity, though, this increase was not significant, compared to the group that only received continuous bGH infusion. However, there was a highly significant increase in the number of positive cells for the same group (100±12.2), compared to group treated with continuous bGH (35±8.6, $P<0.001$), Fig 5.2b. Furthermore, both the mean nuclear staining intensity and number of positive cells was lower in the group that received an acute injection of bGH following continuous bGH infusion, compared to the group that received an acute bGH injection alone (74±1.6 staining intensity and 133±81 positive cells). These results show that the liver remains responsive to an acute GH challenge under continuous bGH exposure during pregnancy. However, as in non-pregnant animals, the liver shows a pattern dependent response to GH, as an acute injection of bGH superimposed on a continuous bGH infusion shows a smaller GH response than an injection of GH without pre-exposure to continuous GH. The continuous pre-treatment thus appears to desensitize the pYStat5 response to an acute challenge .

Figure (5.2) a) Nuclear staining intensity for pYStat5 b) number of positive (pYStat5) cells for liver from pregnant dwarf rats +/- 7 day bGH pump, followed with an acute iv injection of saline or bGH

(a)



(b)



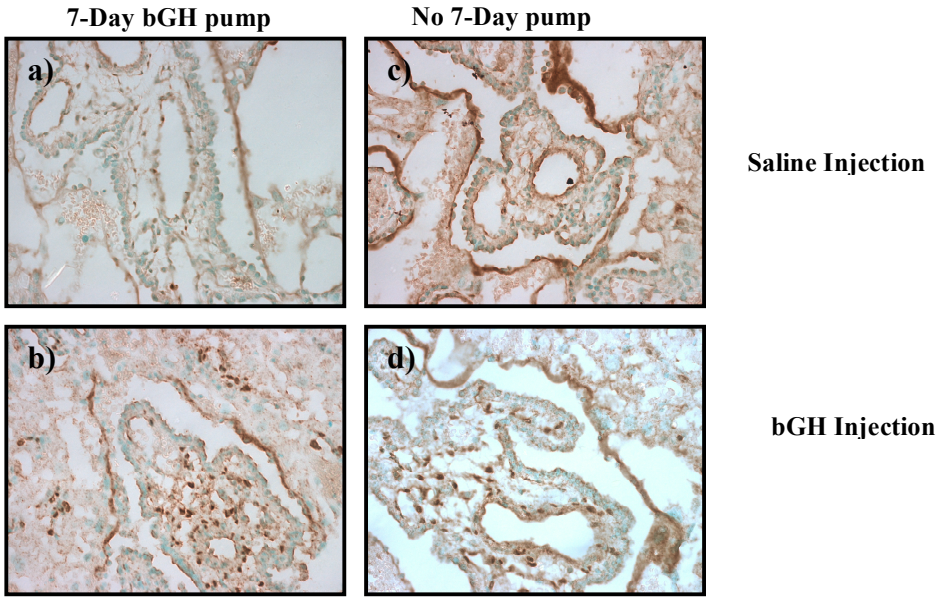
ANOVA followed by Student Newman Keuls test, *P<0.05,

****P<0.001***

5.2.2 Is the placenta also pattern dependent in its response to GH?

Figure 5.3 shows images for the placenta from the same experiments in dwarf rats treated with or without continuous 7-day bGH infusion, and then challenged with an acute injection of saline or bGH.

Figure (5.3) Placental section from dwarf rats, immunostained for phospho Stat5, +/- 7 day bGH pump, followed by an acute iv injection of saline or bGH



pYStat5 staining in the placenta of dwarf rats treated in (a) with continuous bGH infusion (pump) for 7 days and then given an acute injection of saline or (b) with bGH. In the top right, (c) dwarfs received no pretreatment with continuous bGH, but given a single acute injection of saline (c), or bGH in (d). (n=6) for each group.

Figure 5.3(a) shows an example of a placental section from a dwarf rat treated with a 7-day continuous infusion of bGH and then given a single acute injection of saline. I observed a large number of positive nuclei, although they appeared to have a lower level of staining, compared with hepatic responses. Figure 5.3 (b) shows a placental section

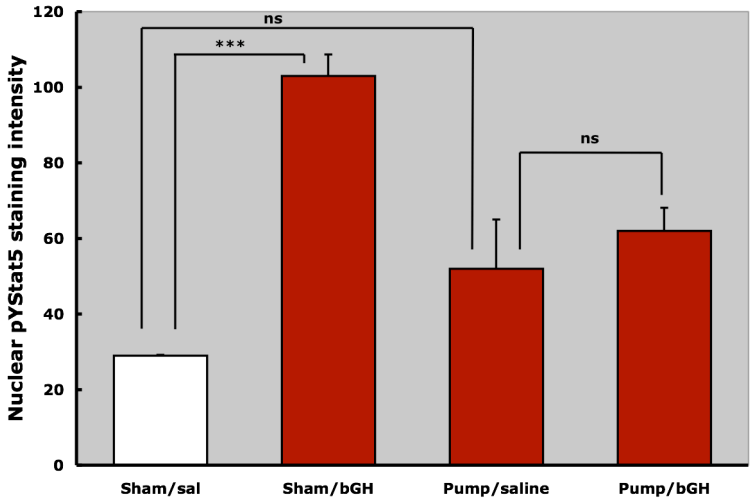
from an animal treated with a 7-day continuous bGH infusion and then a single acute injection of bGH. Several responding nuclei were evident which had a higher staining intensity, when compared to (a). Figure 5.3 (c) shows a placental section from an animal not pretreated with continuous GH, and given only a single acute injection of saline (double control), a few faintly stained pYStat5 nuclei are seen. Again, the strongest pYStat5 response to GH was seen in animals treated with a single acute injection of bGH without continuous GH pretreatment, (Fig 5.3d). Placental images (b) and (d), from the same experiment appear to be very similar in their staining intensity, implying that continuous GH exposure has a lesser effect on placental response to acute GH challenge than in the liver of the same animals, but this needed quantification for all the groups (Fig 5.4)

Figure 5.4a shows a higher mean value for nuclear staining intensity in the placentaes of animals treated with continuous bGH infusion (52±13) compared to saline infused animals (29.0±0.2), but this difference did not reach statistical significance. However, figure 5.4b shows a significant increase in the mean number of positive cells (continuous bGH pretreated 40.0±6.5 vs untreated, 23.1±0.8, $P<0.05$). Giving a acute injection of bGH on top of continuous GH pretreatment, further increased the mean nuclear staining intensity and number of positive cells, but again this was not statistically significant. However, both mean nuclear staining intensity (62.0±6.12) and number of positive cells (52.3±5.3) were lower in the group that received an acute injection of bGH with continous GH pretreatment, compared to the group that had received an acute bGH injection with no pretreatment (103.1±5.7 staining intensity and 58.2±6.5 positive cells).

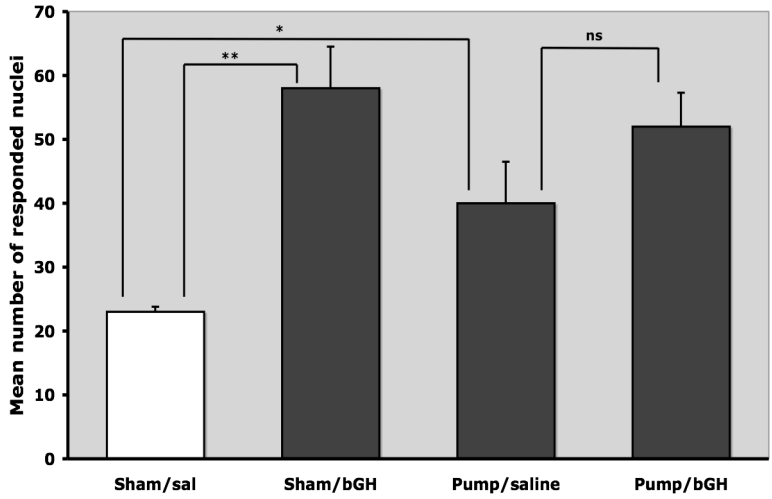
Although there was no significant difference for mean nuclear staining intensity, a significant increase was shown for the mean number of positive cells following continuous GH, suggesting that the placenta (like the liver) is able to respond to GH when given in a pattern of continuous exposure, though less markedly so. This is important as continuous exposure is the more physiologically relevent exposure pattern during pregnancy. Furthermore, as in the liver, the placenta also shows a reduced response to an acute injection of GH following continuous bGH treatment. This suggests that the response to GH in the placenta also shows the same pattern sensitivity as in the liver, although greater pYStat5 responses can be obtained to a pulse of GH. This could occur earlier in human and rodent pregnancies before the pattern is "switched" to continuous.

Figure (5.4) a) Nuclear staining intensity for pYStat5 b) number of positive (pYStat5) cells for liver from pregnant dwarf rats +/- 7 day bGH pump, followed with an acute iv injection of saline or bGH

a)



b)

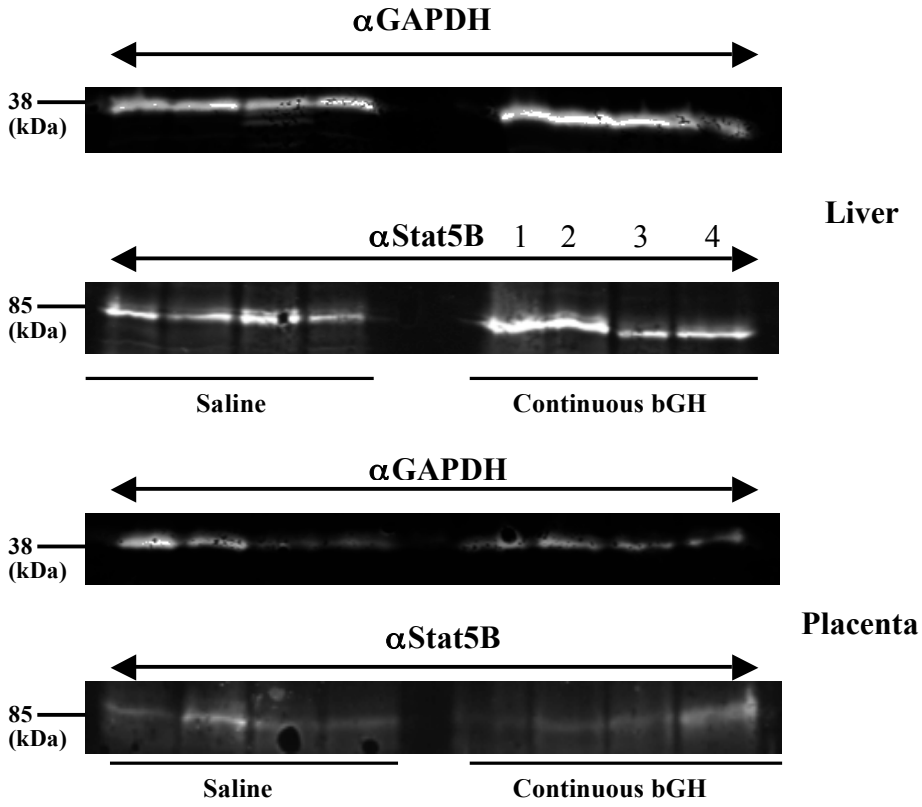


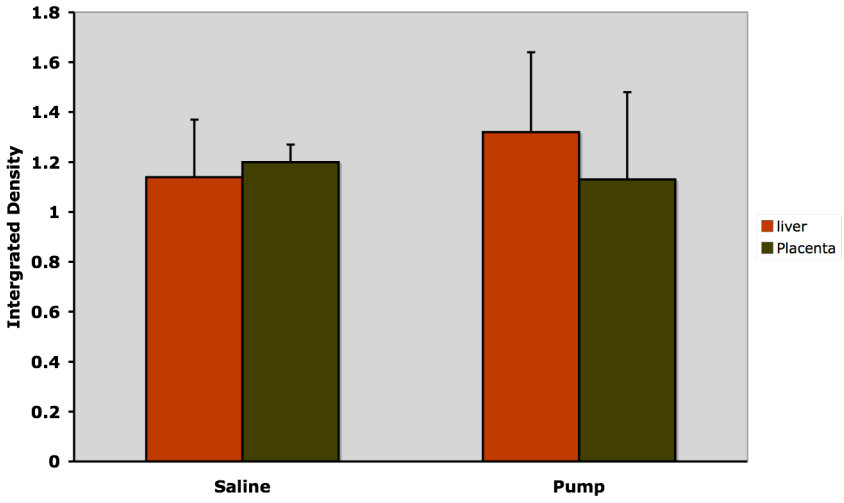
ANOVA followed with Student Newman Keuls test, *P<0.05, **P<0.01, ***P<0.001.

Western blot analysis was carried out in the liver and placenta of pregnant dwarf rats treated with continuous bGH treatment or saline, to asses whether continuous bGH treatment made any changes in endogenous Stat5b, in Stat5b protein levels in the two tissues. Figure 5.5 shows an example of western blots for dwarf liver and placenta treated with continuous bGH or saline. Quantification of the blots for dwarf liver showed no statistical difference in Stat5b levels in continous bGH treated animals compared to saline treated group, when compared with the loading control (GAPDH).

Interestingly, samples 1 and 2 from liver treated with continuous bGH showed stronger bands for Stat5b compared to samples 3 and 4, despite similar loading, suggesting that endogenous Statb levels vary in different animals. Signals were lower in the placenta of dwarf rats, and also showed no differences in groups treated with continuous bGH compared to saline treated, again, possibly due to the variation between animals. Although rather inconclusive it does not appear that GH alters the protein levels substantially in either tissue. Figure 5.5 suggests that there was higher amounts of Stat5b protein in the liver of pregnant dwarf rats treated with continuous bGH compared to the placenta (1.32±0.32 compared to 1.13±0.35, respectively). This could suggest higher steady state levels of Stat5b protein synthesis in the liver. However, it is important to bear in mind that these are Stat5b protein levels at a single time point and this may vary over the course of pregnancy in both tissues. I did not examine this.

Figure (5.5) Effect of continuous GH treatment on Stat5b in the liver and placenta of pregnant dwarf rats





Stat5b in the liver and placenta of pregnant dwarf rats treated with continuous bGH (200µg/day) for 7 days or saline from day 16 of pregnancy.

5.3 The effects of fasting and insulin injections on GH responses during pregnancy

5.4 Introduction

As reviewed in the Introduction, previous studies on the acute insulin like actions of GH have included the ability of GH to decrease blood glucose concentrations, and to stimulate the uptake and transport of glucose in various tissues [133, 350] whereas more long-term, GH has anti-insulin like actions, inhibiting glucose uptake and promoting insulin resistance in tissues. It has been suggested this is due to a convergence and possible crosstalk between the GH and insulin signaling pathways [151, 351] and is quite complex.

A major regulator of insulin is nutrition and several studies have shown both GH and insulin sensitivities vary during calorie restriction [162, 349, 352]. The aims of the experiments described in this section were (i) to look for changes in GH sensitivity (pYStat5 responses) in a known GH-target, the liver, following a 48-hour fast, and (ii) to test whether this was sensitive to the administration of insulin, during continuing of fasting. Preliminary evidence for this interaction had been obtained in normal male rats in the lab by Eveline Gevers (unpublished), so my aim was to see if this could be reproduced in pregnant liver and, or be demonstrated in the placenta. By using GHD models, I could investigate the potential interaction of fasting and insulin with GH signaling in pregnancies without the complication that fasting would altered endogenous GH production, as it does in normal animals.

5.5 GH responses in the liver of non-pregnant dwarf and AS rats, during a 48-hours fast, +/- insulin injection

Previous experiments by Beauloye [162] have shown that a 48-hour fast in male rats is sufficient to induce a state of GH resistance and was also the maximum length of time I was allowed to fast rats on my Home Office Licence. Beauloye showed a reduction in the phosphorylation of both JAK2 and GH-receptors, upon GH administration. Furthermore, the same fasted rats showed only a slight basal phosphorylated Stat5 signal in the liver, when compared to non-fasted animals [162].

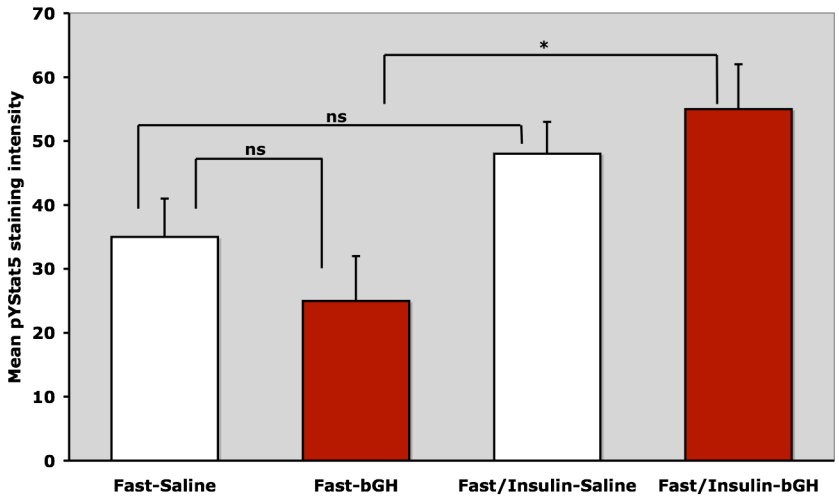
I began with positive controls by carrying out initial experiments in non-pregnant female rats (previous work was done only in males). I used both normal (AS) and GH-deficient (dwarf) female rats, as I wished to establish the effects of fasting on the response to GH, initially in the liver, under normal conditions. I expected clearer responses in GH-deficient rats, and that any effects of fasting and insulin might be most evident in the GH-deficient insulin sensitive condition, compared to intact control females. In subsequent experiments I then compared similar manipulations in pregnant animals, as well as examining placental responses. The dose of insulin administered to animals was chosen based on the observations of Evelien Gevers, and kept the same throughout all experiments. Choosing the correct dose of insulin was important, as I anticipated that after a 48 hour fast, blood glucose levels would be very low, and giving high doses of insulin during continued fast risked the induction of hypoglycemic shock, resulting in fitting and possible death of animals. All experiments were carefully monitored to ensure this did not occur.

Figure 5.6a shows the mean nuclear staining intensity for pYStat5 in liver sections from normal (non-pregnant) fasted AS rats. Remarkably, there was no significant difference in the two fasted groups injected with saline or bGH. This clearly shows that fasting has a major effect to blocking the pYStat5 response to GH in the liver, confirming previous observations in males [291]. It is important to remember that some basal Stat5 activity could be expected in normal rats treated with saline, due to the endogenous GH, independent of what is administered to the animal. However, I would expect this activity to be low, since fasting in rodents causes a reduction in GH secretion (though this has mostly been tested in males) [80]. A much more surprising result was the restoration of a significant increase in mean nuclear staining intensity, following an acute injection of insulin prior to bGH (25±7 vs 55±7, $P<0.05$). Despite continuing fasting, insulin reversed the fasting-induced GH resistance independent of nutrient restoration.

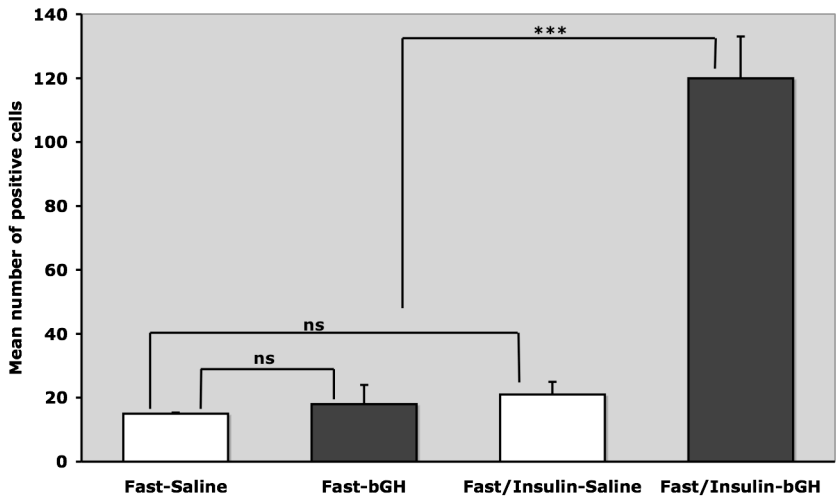
Figure 5.6b shows the number of cells positive for pYStat5 in this experiment. There was no significant difference between the two fasted groups, injected with bGH or saline, confirming that fasting powerfully blocks the GH responsiveness. Again, as for mean nuclear staining intensity, the number of positive cells increased following an acute injection of insulin prior to bGH (15.0±0.5 vs. 120±13, $P<0001$). Clearly therefore, the fasting induced GH resistance in the female liver can be reversed (at least in terms of pYStat5 phosphorylation) by an acute insulin injection.

Figure (5.6) Response to GH in livers of 48 hour fasted female non-pregnant AS rat's +/- Insulin (a) nuclear staining intensity for pYStat5 (b) number of positive (pYStat5) cells

(a)



(b)



Fasting saline vs Fasting bGH, +/- Insulin, *P<0.05, ***P<0.001.

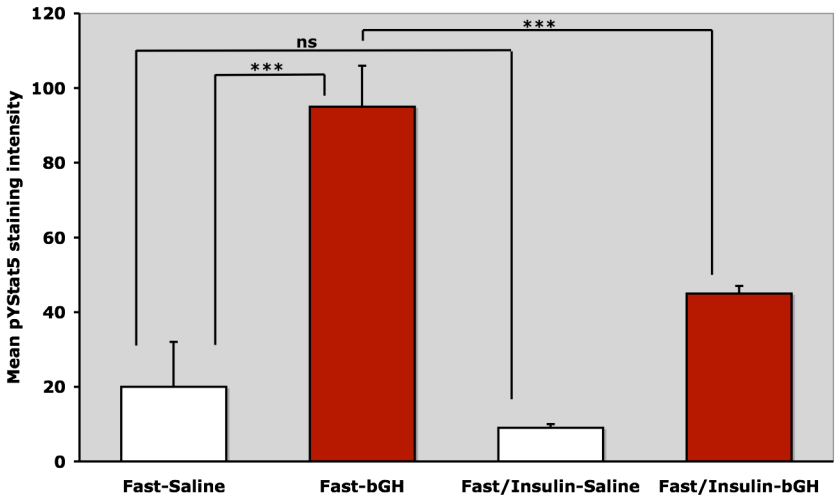
ANOVA followed by Student Newman Keuls test.

Figure 5.7 shows the results of similar experiments, but in female dwarf GHD rats. Here, the nuclear intensity (Fig 5.7a) following GH injection did increase significantly (95±11) when compared to fasted saline injected animals (20±12), $P<0.001$. This was different from the normal animals, and suggests that GHD females are less sensitive to the fasting effects on hepatic GH responsiveness. A further difference was that in these GHD animals an acute injection of insulin resulted in a reduction of mean nuclear staining intensity (45±2), $P<0.001$. This was not a direct effect of insulin as no significant difference in response to GH was shown between fasted saline group and fasted saline groups injected with insulin prior to saline. Figure 5.7b shows the mean number of positive cells measured in the same liver sections. The mean number of positively stained cells in the livers of fasted non-pregnant dwarf rats injected with bGH, was significantly higher (96±20) than that in the saline injected group (7±6, $P<0.001$). Furthermore, as shown with mean nuclear intensity, addition of an insulin injection, prior to bGH, showed a significant reduction in number of positive cells (19±3 vs 96±20, $P<0.001$).

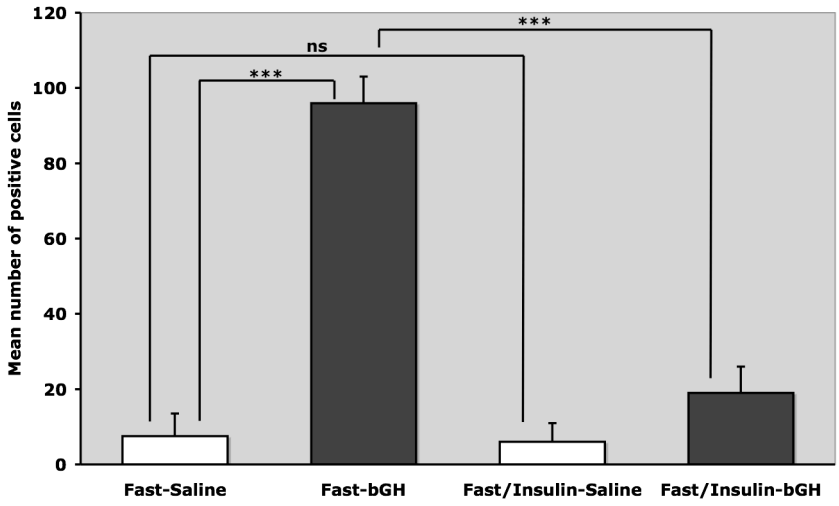
It would appear that the higher GH responsiveness in GHD animals is less sensitive to fasting, and if anything, inhibited by insulin. It is not obvious why such differences are apparent in GHD animals but one possibility is that GHD rats are already more insulin sensitive than normal rats, and their basal nutritional intake is much lower.

Figure (5.7) Response to GH in livers of 48 hour fasted female non-pregnant dwarf rats +/- Insulin (a) nuclear staining intensity for pYStat5 (b) number of positive (pYStat5) cells

(a)



(b)



Fasting saline vs Fasting bGH, +/- Insulin, ***P<0.001.

ANOVA followed by Student Newman Keuls test.

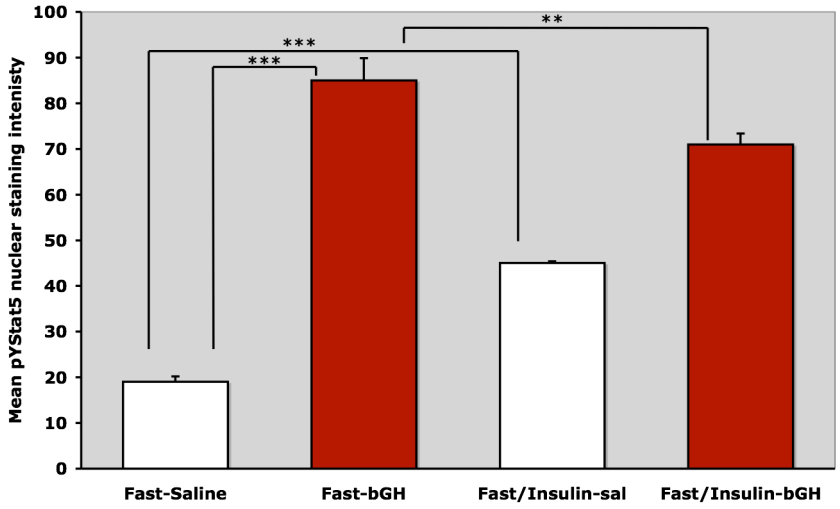
5.5.1 GH responses in the liver of pregnant AS rats during a 48-hour fast, +/- insulin injection

The same fasting experiments were then performed in pregnant rats beginning with normal (i.e. GH-intact) rats. Figure 5.8a shows the mean nuclear staining intensity in liver sections from pregnant wild-type (AS) rats. A significantly higher nuclear pYStat5 staining was observed in fasted rats given a bGH injection (85±5) vs saline injected (19±1, $P<0.001$). Interestingly, an injection of insulin at the 40th hour of a 48-hour fast, prior to an injection of saline, resulted in a significant increase in mean nuclear staining intensity, compared to fasted animals not injected with insulin (45±5 vs 19±1, $P<0.001$). In contrast, insulin pretreatment reduced the mean nuclear staining intensity response to GH (71±2), compared to fasted animals injected with bGH without prior insulin injection (85±5), $P<0.01$. This was reminiscent of the results in the non-pregnant dwarf females. Fasted pregnant rats injected with bGH showed a significantly higher number of pYStat5 responding cells (20±3) compared to saline injected fasting pregnant rats, who had very low basal signaling, (3±08), $P<0.001$. Taken together, this suggests that whilst fasting may reduce the number of cells responding to GH, the response in the *pregnant* liver is diminished to a much lesser extent by fasting, than that in *non-pregnant* AS rats (Fig 5.6a).

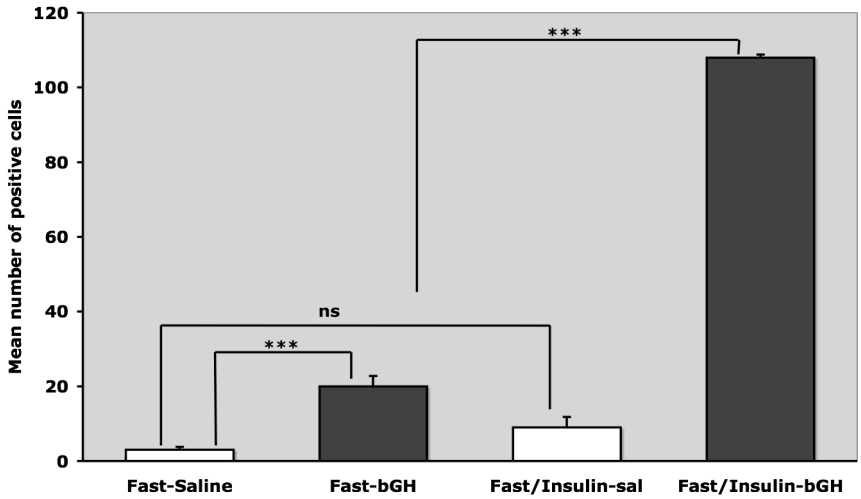
Figure 5.8b shows the results in terms of the number of positive cells. An injection of insulin prior to GH challenge was able to further increase the number of positive cells responding to GH from 20±3 in non-insulin injected group, to 108.1±0.8 in insulin-injected group, $P<0.001$, as in AS non-pregnant rats (Fig 5.6b). No significant increase in these parameters was seen in fasted rats given insulin alone, without a GH challenge.

Figure (5.8) Response to GH in livers of 48 hour fasted pregnant AS rat's +/- Insulin (a) nuclear staining intensity for pYStat5 (b) number of positive (pYStat5) cells

(a)



(b)



Fasting saline vs Fasting bGH, +/- Insulin, **P<0.01, ***P<0.001.

ANOVA followed by Student Newman Keuls test.

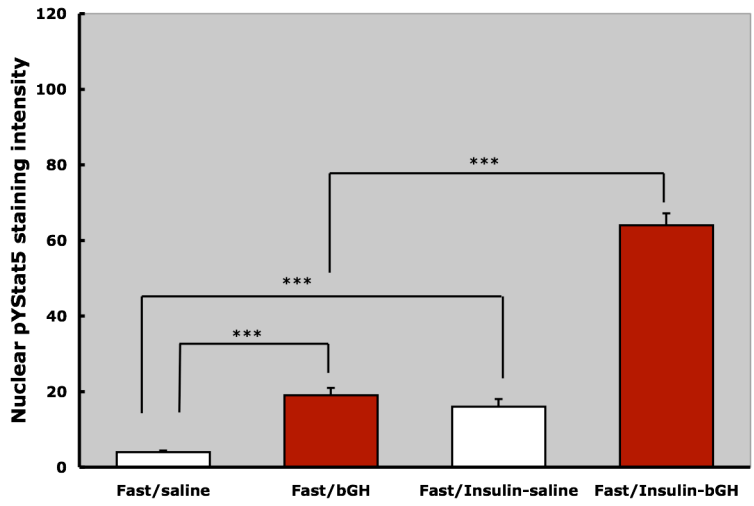
5.5.2 GH responses in the livers of pregnant dwarf rats during a 48-hour fast, +/- insulin injection

The same experiments were then repeated in groups of fasted pregnant GHD *dwarf* rats (Figure 5.9). A significant increase in mean nuclear staining intensity in response to a single injection of GH (19±2) was found compared to saline injected controls (4.1±0.4), $P<0.001$. An injection of insulin without GH challenge also increased nuclear staining (Fig 5.9a) and greatly enhanced the response to GH, (64±3), compared to fasted animals given GH without prior insulin injection (19±2), $P<0.001$. This suggests that in these fasting pregnant GHD rats, insulin pretreatment increases basal GH responses, and has an even greater effect in the fasted state. Figure 5.9b shows the mean number of positive cells recorded for the liver sections of fasted pregnant dwarf rats with essentially the same trends, though the rise in the number of positive cells in the controls, or following an injection of GH did not reach statistical significance. However, an acute injection of insulin prior to GH challenge did produce a significant increase in the number of GH responding cells (117±6), when compared to fasted dwarfs injected with bGH alone (4.0±0.8), $P<0.001$.

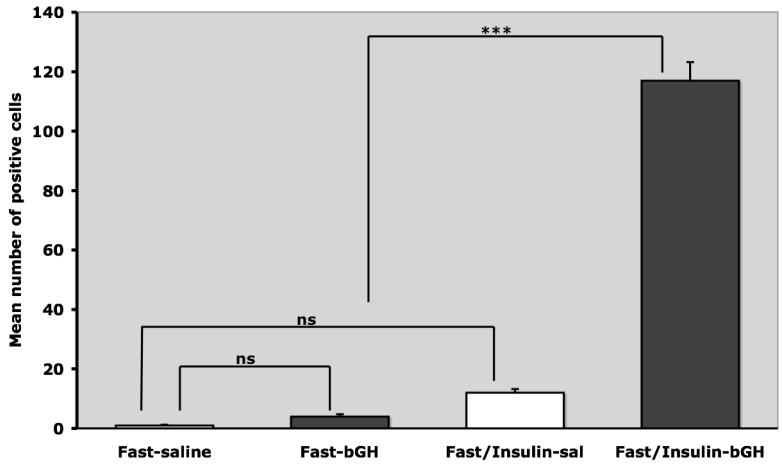
Overall, I interpret my results from these experiments to indicate that giving insulin prior to GH administration during fasting is able to increase the GH responses in the fasting pregnant liver, but the magnitude is highly dependent on prevailing endogenous GH tone. Fasting does reduce pYStat5 responses to GH in both pregnant and non-pregnant liver and is somewhat counteracted by insulin pretreatment, but this response is dependent on the GH-status of the rat.

Figure (5.9) Response to GH in livers of 48 hour fasted pregnant dwarf rats +/- Insulin (a) nuclear staining intensity for pYStat5 (b) number of positive (pYStat5) cells

(a)



(b)



Fasting saline vs Fasting bGH, +/- Insulin, ***P<0.001.

ANOVA followed by Student Newman Keuls test.

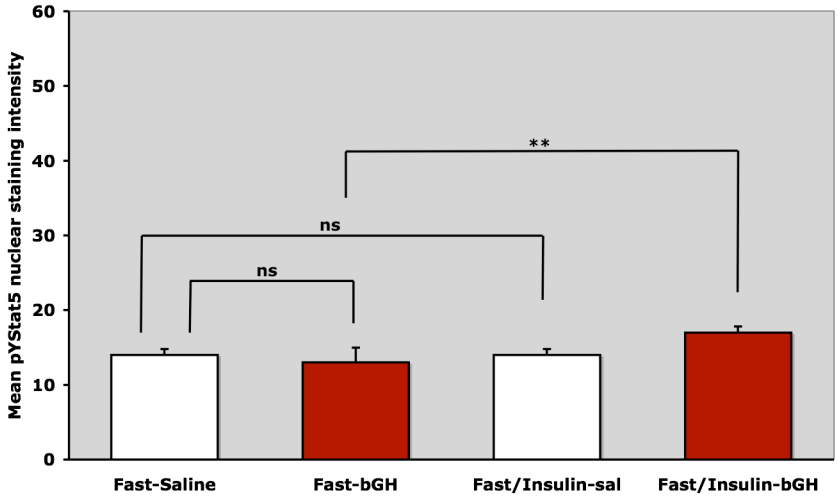
5.5.3 GH responses in the *placenta* of AS and dwarf rats during a 48-hour fast, +/- insulin injection

As I have shown earlier that the placenta is directly responsive to GH, I also tested whether I could detect a similar change in sensitivity to GH in this tissue (like in the liver) following fasting, using placental tissues from the pregnant rats in the experiments described in the previous section. Figure 5.10a shows the mean nuclear staining intensity for placenta from fasted AS rats. There was no significance in pYStat5b responses difference between the fasted groups given bGH or saline, suggesting that, as in liver, the response to GH seen in the normal placenta is lowered by fasting. Injection of insulin had no effect on basal nuclear pYStat5 staining, but significantly increased mean nuclear staining in response to GH, when compared to the fasted group given bGH without insulin pretreatment (13±02 vs 17.0±0.8, $P<0.01$). This effect was relatively small, but nevertheless consistent with what was seen in liver in these rats.

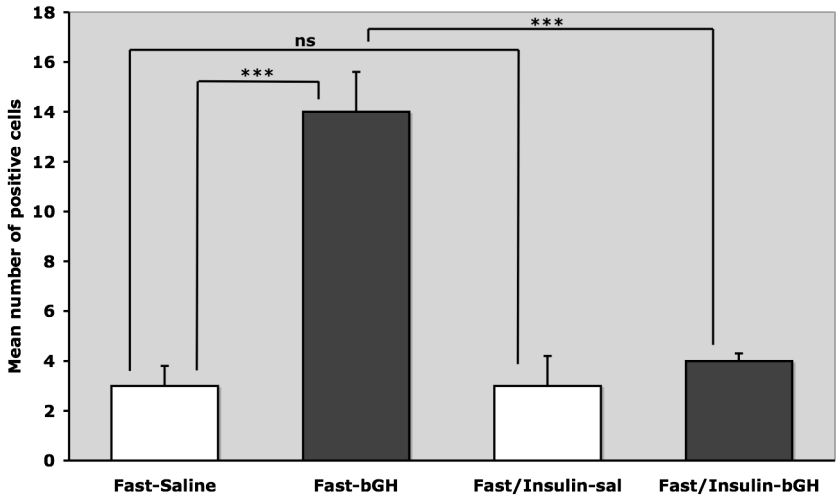
Figure 5.10b shows the number of positive cells recorded for fasted AS rats. There was small but significant increase (14.1±1.6) in the number of cells responding to GH in the fasted placenta compared to saline controls (3.0±0.8), $P<0.001$. This could indicate that whilst fasting reduces the intensity of response to GH in the placenta, some new responding cells, with possibly low staining intensity are still recruited. Interestingly, administration of insulin prior to GH, significantly reduced the number of positive cells (4.0±0.3), $P<0.001$, down to control values. It was unusual to see a divergence between staining intensity and cell number responding, and the reason is not obvious, thought the signals in placenta are much lower overall. No significant changes were observed in the groups given insulin prior to saline challenge.

Figure (5.10) Response to GH in placenta of 48 hour fasted pregnant AS rat's +/- Insulin (a) nuclear staining intensity for pYStat5 (b) number of positive (pYStat5) cells

(a)



(b)



Fasting-saline vs Fasting bGH, +/- Insulin, **P<0.01, ***P<0.001.

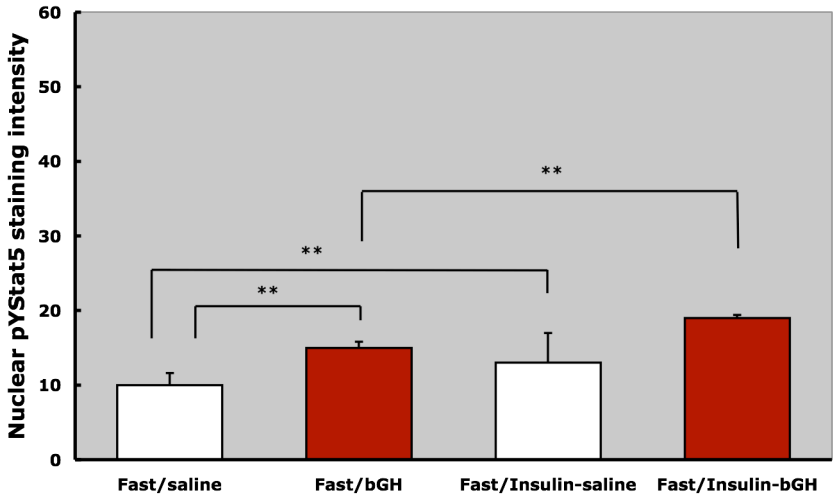
ANOVA followed by Student Newman Keuls test.

I also tested the placental responses to GH in fasting pregnant *dwarf* rats. Again, nuclear pYStat5 staining in dwarf placenta was low, but Figure 6a shows staining intensity to be slightly but significantly higher (15.0±0.8) in fasted animals given GH, compared to saline injected controls (10.1±1.6), $P<0.01$. Figure 5.11a also shows that an acute injection of insulin prior to saline challenge in these fasted animals increased mean nuclear intensity when compared to fasted dwarfs given saline alone (13±4 vs 10.1±1.6, $P<0.01$), and the same was true for GH responses (19.1±0.4 vs 15.0±0.8, $P<0.001$), but the increase was no larger than for saline alone, suggesting the main effect was on basal pYStat5 signaling, rather than GH-induced responses.

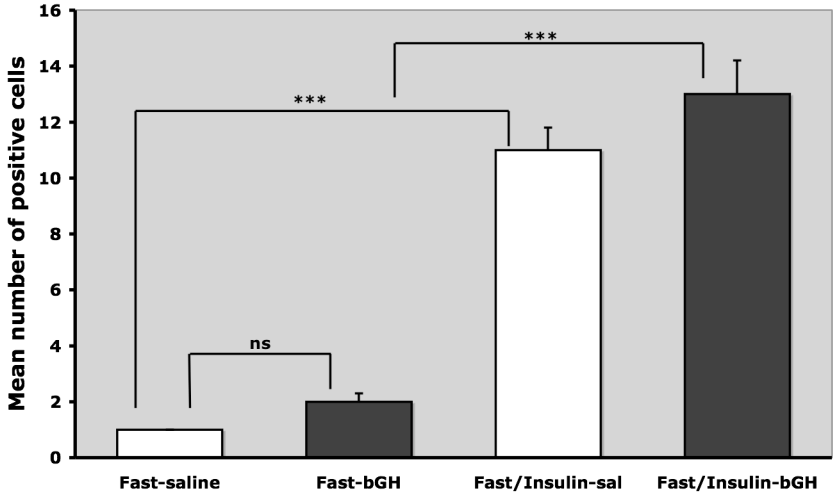
Figure 5.11b shows results for the numbers of positive cells recorded for placenta from these experiments in fasted pregnant dwarf rats. During fasting few cells responded to GH. However, an acute injection of insulin prior to bGH or saline challenge, revealed a marked increase in number of positive cells, in both groups (13±1 and 11±2, compared to 2.1±0.3 and 1±0; $P<0.001$ for both cases) respectively. Again, this implies the main effect of fasting and reversal by insulin is in basal pYSta5 signaling. Despite the small responses, I feel able to conclude for the first time that the placenta from dwarf rats, as from normal AS rats, shows small but significant changes in response to GH following fasting, and that the magnitude of the response is altered by insulin treatment during fasting. However, a major effect in placenta may be in basal pYStat5 signaling, upon which small GH responses are superimposed. It is also important to note that the number of cells responding to GH is fewer in the placenta, compared to the liver. As previously argued, placenta is a very heterogeneous tissue, with only a small area showing cells responsive to GH, relative to the more homogenous liver. Nevertheless, clearly responding cells are clearly present.

Figure (5.11) Response to GH in placenta of 48 hour fasted pregnant dwarf rats +/- Insulin (a) nuclear staining intensity for pYStat5 (b) number of positive (pYStat5) cells

(a)



(b)



Fasting saline vs Fasting bGH, +/- Insulin, **P<0.01, ***P<0.001.

ANOVA followed by Student Newman Keuls test.

5.6 GH responses in the livers of pregnant Non-transgenic (Wild-Type) mice, during a 48-hour fast, +/- insulin injection

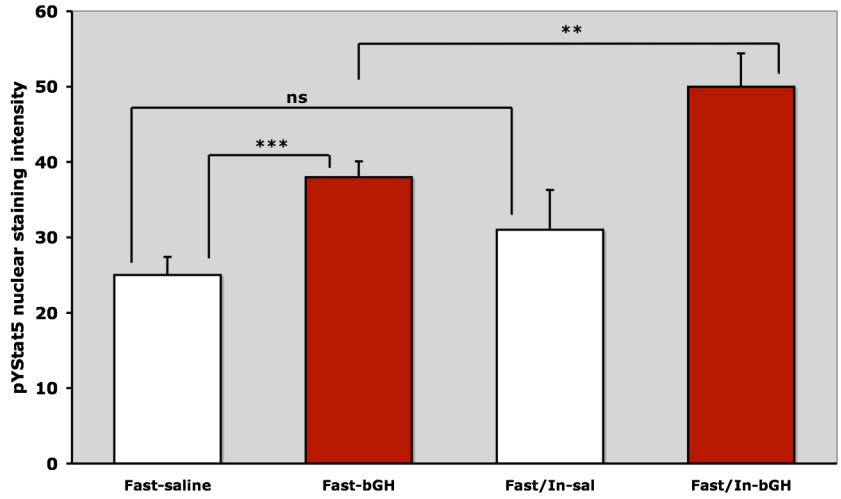
Because of these somewhat divergent results in GHD pregnant rats I decided to repeat similar experiments in another GHD model available in the lab, namely the GH-deficient (GRF-M2) mice. I was curious whether mice would demonstrate a different response to GH, following fasting, compared to rats. Figure 5.12a shows the mean nuclear staining intensity in the liver sections from fasting pregnant wild-type mice. A significant increase in mean nuclear staining intensity in response to a single injection of GH (38.1±2.1) was found, compared to saline injected controls (25.0±2.4, $P<0.001$). Insulin pretreatment before an injection of saline in fasted mice showed no further changes in pYStat5 nuclear staining, however, the insulin pretreatment before an injection bGH showed a significant increase in pYStat5 staining (50.0±4.4 vs 38.1±2.1, $P<0.01$).

Figure 5.12b shows a significant increase in the number of positive cells recorded for fasted animals injected with bGH (138.1±5.7) compared to saline (53.0±4.9), $P<0.001$. However, there was no further increase in the number of positive cells, following pretreatment with insulin prior to saline or bGH, when compared to fasted animals that had received bGH or saline alone. Nevertheless, fasted mice pretreated with insulin prior to bGH showed significantly higher positive cell numbers (136.0±8.5) compared to mice given saline after insulin pretreatment (62.2±5.3), $P<0.001$. These results show that a hepatic pYStat5 response to GH can still be observed during fasting in normal mice, but there is a significant increase in the level of response following insulin treatment, as in rats. However, unlike in my rat

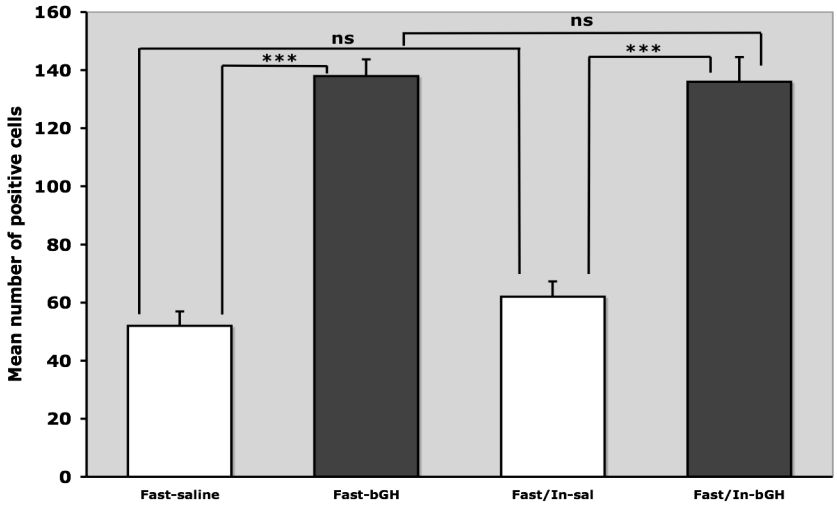
experiments, the number of cells responding was not further increased by insulin pretreatment in these fasting mice.

Figure (5.12) Response to GH in livers of 48 hour fasted pregnant Wild-Type +/- Insulin (a) nuclear staining intensity for pYStat5 (b) number of positive (pYStat5) cells

(a)



(b)



Fasting saline vs Fasting bGH, +/- Insulin, **P<0.01,P<0.001.***

ANOVA followed by Student Newman Keuls test.

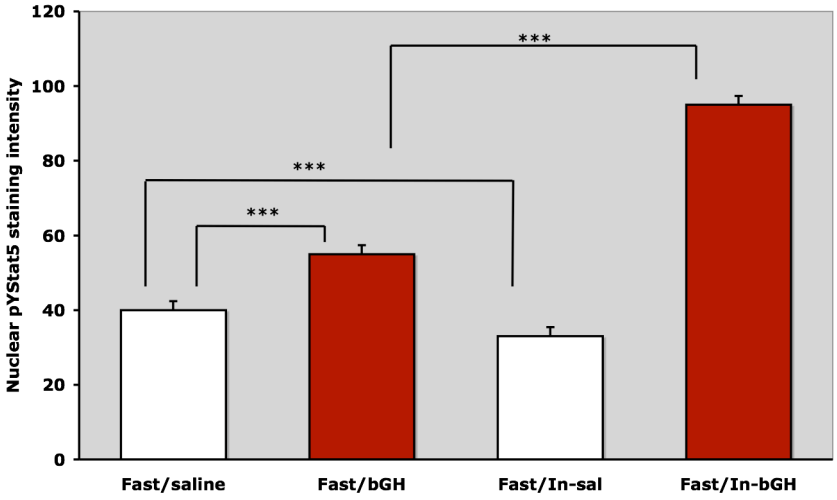
5.6.1 GH responses in the livers of pregnant GRF-M2 mice, during a 48-hours fast, +/- insulin injection

When analogous experiments were repeated in GHD mice, (Figure 5.13), both mean nuclear staining intensity (55±2) and number of positive cells (25±4) in fasted livers of pregnant GRF-M2 mice, injected with bGH was significantly higher than that of saline (40±2 and 14±2, $P<0.001$, $P<0.01$ respectively). Fasting clearly does not abrogate GH-signaling in these GHD mice. However, an injection of insulin, during fasting, 8 hours prior to an injection of bGH, significantly increased both mean nuclear intensity (95±2) and mean number of positive cells (120±2) in the liver, compared to animals injected with bGH alone (55±2 and 25±4, $P<0.001$ in both cases), respectively.

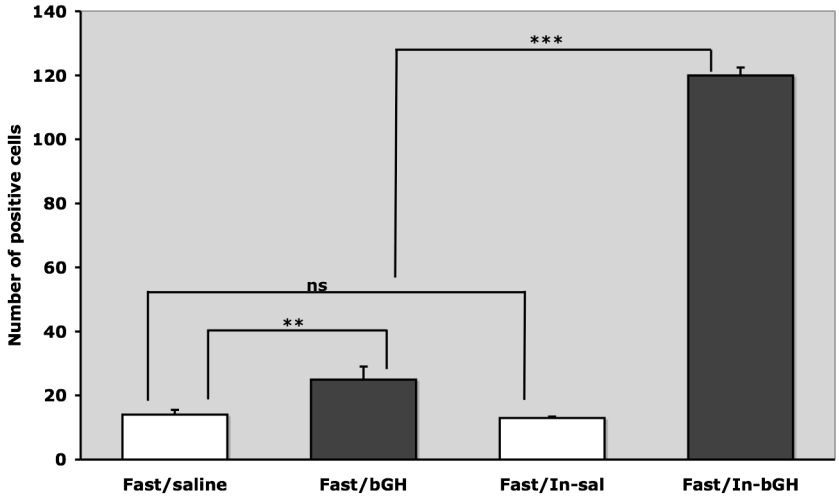
These results are particularly interesting because the responses to GH and insulin induced restoration of GH responses, are more clearly observed in GH-deficient mice, compared to the wild-type littermates. Whilst many explanations may be possible, the simplest is that, as in rats, the prevailing GH exposure from endogenous secretion has a profound effect on GH sensitivity to fasting and insulin reversal. It again emphasizes the importance of prevailing GH tone on the tissue responses to acute challenge with or without nutritional deprivation.

Figure (5.13) Response to GH in livers of 48 hour fasted pregnant GRF-M2 +/- Insulin (a) nuclear staining intensity for pYStat5 (b) number of positive (pYStat5) cells

(a)



(b)



Fasting saline vs Fasting bGH, +/- Insulin, **P<0.01, ***P<0.001.

ANOVA followed by Student Newman Keuls test.

Lastly, I addressed the same important question as in my rat experiments: In the placenta of pregnant mice, could I see GH responses and alterations during fasting and would this differ between normal and GH-deficient pregnant mice?

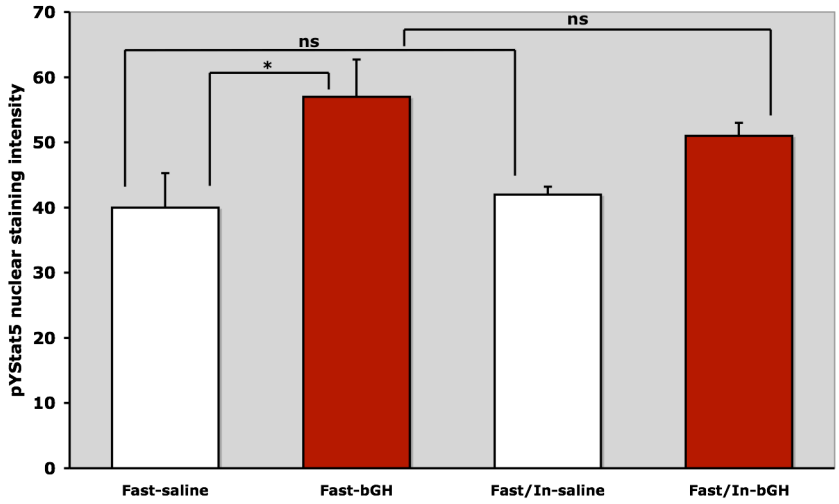
5.6.2 GH responses in the placenta of Non-transgenic (Wild-Type) mice, during a 48-hour fast, +/- insulin injection

Figure 5.14a shows a small but significant increase in mean nuclear staining intensity between fasted normal mice injected with bGH (57±6) compared to saline injected pregnant fasting mice (40±5), $P<0.05$, though no further significant increase in mean nuclear staining intensity was observed in any group following an acute injection of insulin. Figure 5.14b shows a significant increase in positive cell number in fasted mice injected with bGH (71±6), compared with saline injected (47±6), $P<0.05$.

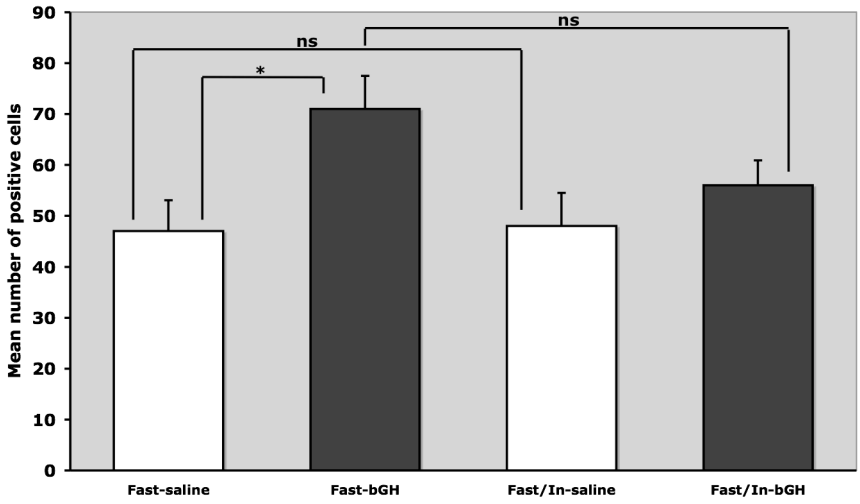
An acute injection of insulin appeared to blunt this GH response, but the data were not significantly different from the group not receiving insulin prior to GH (Fig. 5.14b). Overall, fasting reduced, but did not block the placental response to GH in normal mice, and this was unaffected by insulin pretreatment.

Figure (5.14) Response to GH in placenta of 48 hour fasted Wild-Type mice, +/- Insulin (a) nuclear staining for pYStat5 (b) mean number of positive (pYStat5) cells

(a)



(b)



Fasting saline vs Fasting bGH, +/- Insulin, *P<0.05.

ANOVA followed by Student Newman Keuls test.

5.6.3 GH response in the placenta of GRF-M2 mice, during a 48-hours fast, +/- insulin injection

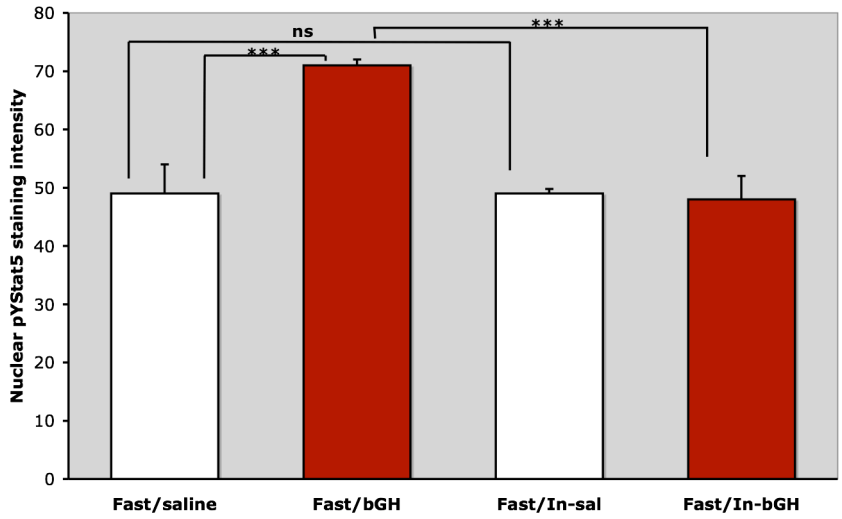
In the placenta from fasting GRF-M2 mice, Figure 5.15a shows that there is a clear GH response in staining intensity (71±1) compared to saline injections (49±5), $P<0.001$. Note that as in the liver responses, the mean nuclear staining intensity is lower, when compared to placenta from non-fasted bGH injected GRF-M2 mice. A highly significant increase was found in number of positive responding cells in the placenta from fasting animals given bGH compared to that of saline (57.1±4.4 vs 13±0.8, $P<0.001$). Again this suggests that GHD background makes it easier to detect GH responses in mice, as in rats, and that fasting does not block this in GHD animals.

An acute injection of insulin during fasting did not increase the mean nuclear staining intensity (48±4), or number of positive cells (21±2), in the placenta compared with fasted mice given bGH without insulin pre-treatment (71±1 and 57.1±4.4), respectively; the response was completely blunted. This was rather unexpected, but similar to that seen in GHD rats, where basal signals went up but additional GH responses were not seen with insulin pretreatment. It is also remarkable that both the nuclear staining intensity and number of positive cells in fasted, bGH injected animals are higher in the placenta compared to the liver in the same fasted mice (71±1 and 57.1±4.4 compared to 55±24 and 25±4, respectively). This suggests to me that in mice, the effects of fasting on blocking GH response are less profound in the placenta than the liver, and might explain why the restorative effects of insulin are less clearly observable in this tissue.

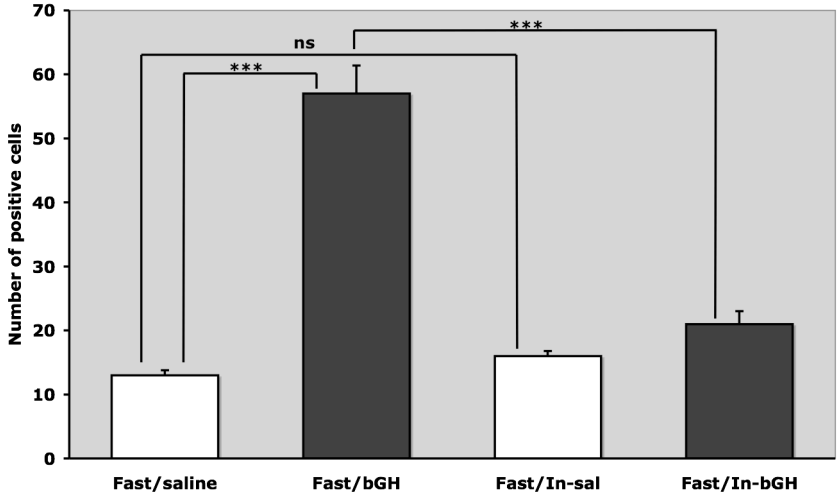
Figure (5.15) Response to GH in placenta of 48 hour fasted GRF-M2, +/- Insulin

(a) nuclear staining intensity for pYStat5 (b) number of positive (pYStat5) cells

(a)



(b)



Fasting saline vs Fasting bGH, +/- Insulin, ***P<0.001.

ANOVA followed by Student Newman Keuls test.

5.7 Discussion

(i) GH patterns

Results from this experiment confirmed that a pulsatile pattern of GH is more effective in activating phosphorylation of Stat5 than a more continuous pattern [353] but this is shown here for the first time in the placenta. My results also indicate that both the liver and placenta continue to be responsive to GH during pregnancy, regardless of the pattern of GH given. They also show that the liver during pregnancy (as in non-pregnant rat [291]) shows the highest level of Stat5 activity when given a pulse of GH. Liver Stat5b is known to be strongly and repeatedly activated in response to incoming plasma GH pulses [353], while a more continuous presence of GH leads to substantial down regulation of Jak2-dependent signaling to Stat5b [354]. However, earlier studies have shown cytoplasmic Stat5b and Jak2 expression to be similar in female and male liver [355, 356]. I showed that a single injection of GH is able to evoke a higher Stat5 response, in the liver of untreated pregnant dwarf rats, compared to those pre-treated with continuous GH infusion. This is true for both mean nuclear staining intensity and the number of positive cells. The reduction in the both mean nuclear staining intensity and the number of positive cell in the group treated with continuous bGH with or without an acute injection of bGH, suggests that continuous GH exposure down-regulates the Stat5 response to an acute challenge with GH.

Mine are the first experiments to show that the placenta is also able to respond to GH, with continuous exposure. Like in the liver, the placenta from dwarf rats also appears to show higher Stat5 activity in response to a single acute injection of GH, compared to continuous GH infusion, for both mean nuclear staining intensity and number of

positive cells. The quantification in placenta only showed a statistically significant difference for the number of positive cells, when comparing animals pretreated vs not pretreated with continuous GH. This might be explained by smaller signals relative to higher background in this tissue. As mentioned earlier, some cells with very low staining intensity could be discarded during the background subtraction process. It is also important to note that this method used to detect responding nuclei and levels of intensity seems to give clearest results with GH pulse exposure, where the acute response can be easily distinguished above background. In contrast, in groups pretreated with continuous GH the increment of further pYStat5 nuclear staining in response to an acute challenge is smaller, making it more difficult to detect changes in the intensity of signal after background subtraction.

The importance of a GH pulsatile pattern has been outlined in previous studies [63, 357] with particular emphasis on its effectiveness on stimulating weight gain and body growth. However, the importance of continuous GH secretory pattern seen in female rodents is less clear. The potential effects of different GH patterns on the placenta and possible role during pregnancy, remains to be elucidated, but are likely to be most relevant for continuous exposure. Given the weaker responses, it is likely to be less powerful than in the liver, at least from circulating GH. Whether locally produced continuous GH exposure is more effective on the placenta in humans remains an attractive possibility, but would not be the case in rodents that lack placental GH. Concentrations in human placenta will be much higher than those reaching the liver after dilution in the circulation.

Both hepatic GH receptors and plasma GHBP levels are sensitive to the pattern of GH exposure. Male rats show much lower levels of GH receptor expression as well as GHBP levels when compared to females [107]. Furthermore, pulsatile GH infusion has very little effect on GH receptor expression, whereas continuous GH exposure leads to increased GH receptor and increased GHBP levels [54, 55, 357]. These findings suggest that (i) continuous GH exposure doesn't impair the ability of the liver to respond to GH, as there isn't any reduction in GH receptors, and (ii) up-regulation of GH receptor expression and GHBP levels may be a mechanism to deal with the changes in GH pattern. However, there is a paradox that higher levels of GHR in female rat liver are associated with reduced responses and growth rate. Nevertheless, continuous GH given to hypophysectomized rats, although not being as effective as administration of pulses, does stimulate appropriate growth [48]. Some authors have suggested that high GHBP levels may act as a method of protecting tissues from high levels of GH that may not be beneficial, by competitive binding of GH to reduce the signaling effect of GH [358, 359]. Conversely, high levels of GHBP could also serve as a reservoir to prolong the time of low-level GH exposure [360] reducing GH clearance. Fairhall *et al* (1992) [111] shows that pre-incubation of recombinant human GH and human GHBP, 60 minutes before being injected into guinea pigs, also prolongs the *in vivo* clearance of both proteins greatly.

However, it is important to remember that female rats do grow normally, and do show some pulses, these being more frequent and irregular than in males [79, 361] superimposed over a higher baseline. This more continuous irregular pattern of GH exposure is clearly growth promoting in females. Other studies carried out in the rat have shown that continuous GH exposure at various baseline levels can be more

involved in the metabolic actions of GH and regulating the expression of several GH-sensitive proteins [76, 362] and to increase lipolysis [139]. Nevertheless, there has been no study that has looked at pattern dependent effects of GH on GH receptors and gene expression during pregnancy, the only physiological condition in which GH secretory pattern is notably more continuous. Such experiments could help with understanding of the potential functions of a more continuous pattern of GH on, for example, metabolism in pregnancy.

(ii) Nutrition and Insulin

A number of studies in fasted rats report a dramatic reduction of GH release [80, 159, 363] along with a state of GH resistance, which is responsible for a reduction in IGF-1 levels [161]. My results obtained from fasted non-pregnant, AS rats were consistent with these findings, in that I showed a state of GH resistance, with reduced hepatic GH responses. The results for non-pregnant dwarf rats did not parallel those obtained in AS rats. The dampening effects of fasting on hepatic GH response was not as clear in the non-pregnant dwarf rats. Although, there was a reduction in hepatic GH response compared to non-fasted female dwarf rats, a significant response to a challenge of GH is still observed with an increase in pYStat5 during fasting. This was a surprising result, as reported in a previous study, GHR mRNA levels in dwarf liver are significantly lower than in normal rats [364] so one might have expected there to be very low hepatic GH responses in dwarf rats during fasting compared to normal rats. However, hepatic GHR binding (i.e. protein level) in dwarf rats are similar to normal rats [364, 365]. Furthermore, although dwarf rats produce smaller amounts of GH, they still maintain a sexually dimorphic secretory pattern [301] and are very sensitive to low doses of GH. This would suggest that the GH deficiency doesn't

affect the sensitivity of hepatic GH receptors to GH, and that dwarf tissue are adapted to low levels of GH occupancy and signaling. The reduction of hepatic GH response in both the normal and dwarf rats during fasting implies a reduction in GH binding, under these circumstances. This is in agreement with other studies that show that nutritional deprivation causes decreased GH binding in the liver [163, 365] in rats.

Since hepatic GH receptors are sensitive to GH exposure patterns, another explanation for the observed GH response with such low levels of GH in dwarf rats could imply that hepatic GH receptors are sensitive to these differences in the plasma GH pattern at low circulatory GH concentrations. So the amount of GH required, to evoke a hepatic response in dwarfs to an acute pulse might be much lower than in normal rats. This would also imply that rather than simply the number of GH receptors, the level of GH response may be determined by other factors, such as the rate in which GH receptors become coupled to Jak2, or their turnover. The mechanisms involved in the down-regulation of the different components of the GH signaling pathway are not entirely clear, however, in fasted male rats GH stimulated JAK2 phosphorylation is severely blunted [162] with only a slight phosphorylated Stat5 signal being observed. Furthermore, over-expressed SOCS3 blunts Jak-Stat activation [366] and has been described in cases of fasting and GH resistance [367, 368]. It is also important to note that it is more difficult to assess the level of repression of endogenous GH response caused by fasting in dwarf rats, as the level of basal GH is already very low prior to fasting in these GHD animals.

In the same experiments pretreatment with insulin prior to a GH challenge during fasting showed restored hepatic GH response in the liver of dwarf rats. It is well accepted that GH modulates tissue responses to insulin [133, 143]. GH deficiency is associated with increased insulin sensitivity, decreased insulin secretion and decreased fasting glucose concentrations [144, 145]. Nevertheless, it is remarkable that a single insulin pretreatment can reverse the inhibiting effects of fasting, despite continued nutritional deprivation. Insulin pretreatment in both the pregnant normal and dwarf rats was able to reverse the effects of fasting, showing an increase in the number of GH responding cells when given prior to GH. Some of the metabolic roles of GH promote gluconeogenesis and lipolysis, resulting in the release of glucose and energy sources to the body. I envisage there could be high demand for this during fasting in pregnancy, for fetal growth and maternal health. The direct effects of insulin are opposite to GH, in that it promotes the storage of glucose as glycogen in the liver, and inhibits lipolysis.

The crosstalk of insulin and GH signaling via phosphorylated substrates could result in insulin enhancing GH-initiated signaling, resulting in the activation of GH like metabolism and a reduction in GH resistance. One explanation for the restoration of hepatic GH responses only in the dwarfs could be due to the increase in the abundance of insulin receptor (IR) in their liver, which could imply a receptor up regulation due to hypoinsulinemia. This is supported by a study that shows increases in insulin receptor abundance in GHR knockout mice [150]. In contrast, exposure to chronic GH excess leads to hyperinsulinemia, which in turn produces a down-regulation of IR in liver, shown in transgenic mice over expressing GH [369]. I suspect that the restorative effect of insulin I observed during fasting is somehow

exerted on the cross talk between the GH and insulin pathways, rather than simply enhancing nutrient uptake, since fasting continued throughout any experiments. Both *in vitro* and *in vivo* studies show that GH can promote the tyrosine phosphorylation of IRS-1 and IRS-2 and their association with PI 3-kinase [146-149]. Furthermore, studies have also shown Stat5b to be a substrate for the insulin receptor [370]. Chen *et al* (1997) [371] identified Stat5b as a substrate of the insulin receptor using the yeast two-hybrid system and found that Stat5b interacts through its SH2 domain with receptor phosphotyrosine 960 localized in the cytoplasmic domain of the insulin receptor. The extent of such cross-talk could well depend on prevailing endogenous GH status, which might go some way to explain the dependence of this insulin effect during fasting on the GH status of the models used. For example the same observations made in the liver of normal pregnant rats showed that fasting had less of an effect in diminishing the hepatic GH response compared to non-pregnant fasted rats. Furthermore, hepatic GH responses were still evident in fasted pregnant *dwarf* rats.

Many other factors could regulate this, one of which is estrogen, a major regulator of GH binding in the liver. Female rats have 2-3 fold more liver GH binding sites than male rats [365] with estradiol implants in male rats resulting in the increase of liver GHR expression and GH binding protein [114]. Estrogen production increases during pregnancy [372] and may result in an up-regulation of GHR expression in the liver, thus altering the effects of fasting in pregnancy. This could also be something particularly more pronounced in females as most experiments that have looked at GHR expression in fasted rats, report a reduction of GHR mRNA [161, 373]. It is unclear how estrogen and fasting would interact.

My results showed the placental response to GH in both normal and dwarf rats were sensitive to fasting. The reduction in GH response was particularly evident in the dwarf placenta, in which no new pYStat5 positive cells were seen following a GH challenge. Nutrition is obviously important for the progression of normal pregnancy [374] and the placenta has a central role in nutrient transfer, as well as being a source of IGF-1 production [375, 376], therefore I thought that nutritional restriction could potentially have potent effects on GH responses in the placenta. Under fasting, an interesting observation was that the level of signal recorded from responding cells, was generally more faint, compared to non-fasted animals. This was to be expected if the level of response to GH was reduced due to GH resistance. An explanation for the GH resistance observed in the placenta as well as the liver could be due to the down regulation of Jak2 initiated phosphorylation and the up regulation of suppressors of cytokine activity. A study by Miquet *et al* (2005) [390] showed a 3-fold increase in cytokine-induced suppressor (CIS) protein content in the liver of pregnant mice, compared to virgin animals. CIS has been implicated in the desensitization of GH-Stat5b signaling by continuous GH levels [377] and has also been considered a post receptor mechanism partially responsible for hepatic GH resistance shown in the later part of rodent pregnancy, when elevated GH and hepatic GHR expression is accompanied with a reduction in hepatic IGF-1 production [313].

It was important to test my results by fasting another rodent GHD species. Both normal and GH deficient (GRF-M2) pregnant mice showed a reduced GH hepatic response compared to non-fasted animals, however, both normal and GRF-M2 mice do still respond to an acute challenge of GH. An interesting observation was that

insulin in GRF-M2 mice (as in dwarfs rats) appeared to restore the GH hepatic response more markedly than in normal mice. This adds further support to my idea that reversibility of the effects of fasting by insulin on hepatic GH response could be more evident in a GHD background. Since insulin sensitivity is clearly elevated in conditions of low GH, it is more likely that the GHD rodents are more sensitive to insulin and thus the response to the pretreatment is more marked than in normal rodents whilst the GH response in the placenta of normal and GHD mice were lower than in to non-fasted animals, they were still able to show a response to an acute GH challenge.

Interestingly, in both normal and GHD deficient mice the *placental* response to GH was blunted by insulin pretreatment. Insulin resistance in normal human pregnancy is a critical physiological adaptation designed to limit maternal glucose uptake, to ensure that an adequate supply of nutrients is shunted to the growing fetus [378]. The resistance of the placenta to insulin pretreatment in my results may be an example of this protective mechanism. Furthermore, transgenic mice over expressing human placental growth hormone in the liver show severe insulin resistance with insulin levels 4- to 7-fold greater than those in their wild type littermates [379]. On the other hand, I had to consider that I only used a single dose of insulin, and it may have not been as appropriate in the GHD animals as in GH intact animals.

The hepatic GH responses in general were not so dissimilar between rats and mice. What stands out in my experiments is that the GH responses were reduced in pregnant GHD rodents during fasting compared to normal counterparts. This could be explained simply in the difference in their body size. The GHD rodents have smaller

body mass, less energy reserves compared to normal rodents. This difference could be another factor that dictates the severity of the effects of fasting, causing a higher level of GH resistance in a GHD background. Smaller animals generally have a higher relative body mass reduction during fasting compared to larger animals, normal mice show a 7% reduction in body mass, compared to 5% reduction in normal rats [380]. Another interesting finding in my results is that the placental response to GH was less affected by fasting in mice than in rats. This was a surprise to me as the smaller body size of mice led me to believe that the placenta would be more affected by fasting. Studies that assess the effects of fasting on placenta in different sized animals are scarce, but it appears that the placenta in my mice experiments was less affected by effects of fasting, at least in terms of acute pYStat5 response to GH. GHD mice have less food intake than normal mice, so restriction may have different effects on nutrient partitioning via the placenta, for the purpose of protecting the fetus from the effects of fasting. Is this why the effects of fasting appear less evident on the GH response in the placenta of GHD mice, compared to maternal liver?

In summary, I have for the first time examined the effects of fasting on GH sensitivity, in the pregnant liver and the placenta of rats and mice. Furthermore, I showed that the administration of a single acute injection of insulin is able to restore the response to GH in these tissues under the continuing state of fasting in normal, but not GHD rodent placenta. This demonstrates the crosstalk between the GH and insulin signaling pathways, which in turn, could mediate the up-regulation of local IGF-1 generation, counteracting the fall of IGF-1 caused by fasting and GH resistance. The system however, shows quite variable responses, and one key factor is the GH status in the different models, I used.

6. Placental gene expression in pregnant dwarf rats treated with continuous GH or acute GH injection: A preliminary Microarray study

6.1 Introduction

GH is thought to exert many of its effects on cellular metabolism, proliferation and differentiation by regulation of gene expression. My work so far has been limited to the initial events in the GH signaling cascade. For biological responses, clearly there must be downstream targets of the GH-induced pYStat5 response. Whilst it is possible that the range of effects on placenta may be similar to those in other tissues (e.g. metabolic, lipid mobilizing, protein anabolic, insulin antagonizing), in the time available, I decided to concentrate on a preliminary approach to identifying, potential initial direct gene targets for GH. The possibility to use microarray to explore expression data of thousands of genes, across a multiple of experimental paradigms, was the obvious route to pursue. Numerous microarray studies have already been carried out to analyze changes in gene expression in rat liver following GH treatment. Most of these experimental designs have focused on gender and the action of specific hormones on gene expression profiles [381-383]. I decided to use this approach to try to identify preliminary potential candidate targets for direct GH action in the placenta.

In this chapter, I present my preliminary microarray data and analysis of gene expression profiles obtained for dwarf rat placenta, following administration of GH to pregnant GHD animals. Two objectives of this work were (i) to characterize the effects of GH on global gene expression in the placenta, and (ii) to try to identify GH-

responsive genes that might be sensitive to the GH secretory pattern. Many GH regulated genes have been identified from studies of liver tissue from rats deficient in GH, which have provided information on the consequence of a lack of GH on gene expression, highlighting candidate genes that may be directly or indirectly regulated by GH. Therefore using GH-deficient rats I manipulated the pattern of GH exposure, by continuous vs intermittent GH treatment in an attempt to identify genes responding to GH, in a pattern specific manner. Administration of continuous GH infusion to normal pregnant rats (likely having an continuous endogenous secretory pattern of GH) would create a state in which GH continuous signaling would be in excess. In these conditions, changes in gene expression would be a result of abnormally increased GH levels, rather than continuing pattern *per se*, but might have increased the chances of detecting weakly responding genes.

I took three directed approaches to analyze my results, using both biased and unbiased approaches. First, I focused on genes known from the literature to be regulated by GH in other tissues. Second, I sought to identify genes *in silico*, reported to be expressed in placenta, to see if any had identifiable Stat5b response elements, as these could reasonably be direct targets for GH actions. Finally, I used standard software to identify those genes that showed the greatest change in response to GH (which could of course be secondarily affected by GH), using a more unbiased approach.

6.2 Animals and experimental treatments

Three groups of three pregnant dwarf rats were used, of which group 1 was the control, injected with saline only, group 2 received two individual intravenous injections of 100μg bGH/100g bodyweight, 24 hours apart, and group 3 received 48 hour continuous bGH infusion by subcutaneous osmotic mini pump. The bGH

infusion was given at 200μg/day for 48hrs, as my previous experiments have shown that a 200μg/day infusion of GH is an adequate dose to achieve a pYStat5 response to GH in the placenta; this was also the dose previously used by Gevers *et al* (1996) [78]. The time duration for culling of 48 hours was set for all treatment groups, as it was considered a reasonable time to expect to see significant transcriptional changes. The bGH injected group 2, was treated with two acute injections of bGH, as I was concerned that the GHD state may require a first injection to activate the GH signaling cascade i.e. recruitment of transcription factors and phophorylation events, and thus the second injection might be predicted to activate more downstream events such as gene transcription, once the cells were primed with factors recruited with the initial GH injection. Finally due to cost constraints, I restricted the experiment to three animals per experimental group, which should be an adequate number to allow for some variation amongst individual animals but I hoped would still show the strongest consistent responses. Four hours after the final GH injection or 48hrs after infusion, animals were culled, placentae dissected and stored at -80 until RNA extraction was performed (see methods). Extracted RNA was sent to the array services at the Institute of Child Health, UCL, by Affymetrix analysis. Data files generated for gene transcript levels were then returned to me and initially processed by our local bioformatics expert, Dr A Sesay, who kindly assisted me with the analysis of these studies, using his expertise with GeneSpring software (GX 9.0). Table 6.1 lists some well-characterized GH regulated genes and the evidence/studies that were the source of this data. It is important to note that the well-characterized GH target genes in Table (6.1) are widely diverse in their documented functions. I felt it possible that any identified genes to be responsive to GH in the placenta, may also follow a similar diverse trend of functions.

Table (6.1) Known GH- target genes identified in GH-deficient rat liver

GENE APPREVIATION	FULL NAME	REFERENCE/FUNCTION
IGF-1	Insulin-like growth factor 1	Mediator of the growth-promoting effects of growth hormone, Le-Roith *et al*., 2001
IGFBP-3	Insulin-like growth factor binding protein 3	Modulator of IGF bioactivity, Ferry *et al*., 1999.
ALS	Acid-labile subunit	Stabilizer of IGFBP3/IGF complex in the vascular compartments and extends the half-life of IGFs in the circulation, Leong *et al*., 1992.
FABP	Fatty Acid Binding Protein	Cytosolic proteins that enhance intracellular transfer of fatty acids and involved in the processes of fatty acid metabolism, Bennett *et al*., 1994.
Spi2.1	Serine peptidase inhibitor, clade A, member 2.1	Inhibitory actions on caspase-independent cell death.
EGFR	Epidermal Growth Factor Receptor	Cell signaling molecules involved in diverse cellular functions, including cell proliferation, differentiation, motility, and survival, and in tissue development, Wang *et al*., 2004.
SOCS	Suppressor of cytokine signaling	Negatively regulate this signal transduction, Hilton *et al*., 1998.
c-fos	c-fos gene	Growth related transcriptional control.
c-fun	Protooncogene	-
IRF-1	Interferon Regulatory Factor 1	Regulation of type I interferon gene expression, Myamoto *et al*., 1988.
HNF-α	Hepatocyte Nuclear Factor-1 alpha	Required for hepatocyte-specific transcription of the genes, Courtois *et al*., 1987.
Fibrinogen β		Blood plasma protein that plays a crucial role in hemostasis, Gonçalves *et al*., 2006.
Gap130		Signal transducer, associated with JAK1, Lutticken et al, 1994.
Stat3	Signal transducer and activator of transcription 3	Transcription factor that plays a role in induction of gene expression during acute phase response, Akira *et al*., 1994.
p28MAPK	-	-
GADD45	Growth Arrest and DNA damage –Inducible gene	Involved in DNA replication and repair.
APEN		-
MTI-MMP	Membrane type-1 matrix metalloproteinase	Remodeling of tissue, McCawley *et al*., 2001.
MCT1	Monocarboxylate transporter 1	Up regulation of transporters involved in brain metabolism, Cains *et al*., 2008
18S	Tetrapod ribosomal RNA	-

The lists of genes shown have been identified in the rat liver as a result of rapid increases in mRNA, following GH administration, (Mathews et al., 1986, Lemmey et al., 1997 and Ooi et al., 1997). Genes in bold have more recently been identified, also in the liver, to be upregulated by GH, (Barry et al., 2002).

6.2.1 Micoarray data normalization and Quality control

The initial part of the microarray analysis required normalization and quality control of all gene array data, combining the intensities of multiple probes within a probe set, across all chips into one measure of gene expression, and attempts to compensate for systematic technical differences between chips. My analysis used the PLIER algorithm, to generate a single summarized value for expression of genes represented on a chip by several probe sets, (comprising perfect match (PM) or mis-match (MM) probe sets). PLIER separately measures the strength of both (PM) and (MM) probe sets, applies dynamic weighting of the most informative value probes, resulting in a single value for each probe set, with the subtraction of (MM) probe sets.

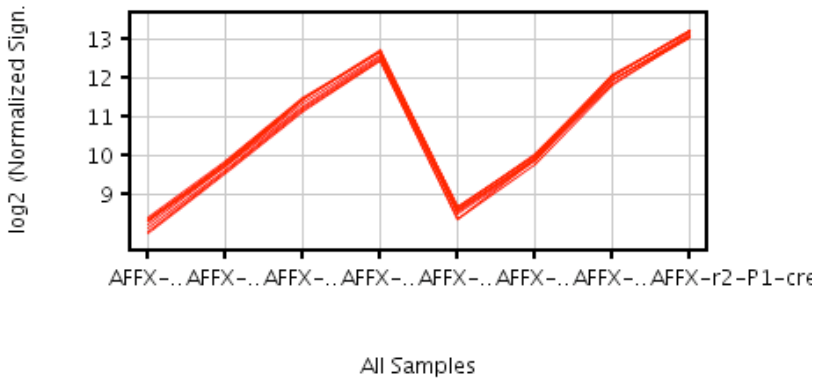
The advantages of PLIER, is (i) a higher reproducibility of signal (lower coefficient of variation) without the loss of accuracy, and (ii) it retains accuracy and higher differential sensitivity for genes with lower expression values.

Using the integral Affymetrix GeneChip quality controls two separate assessments were performed: assessment of known hybridization controls on the chip, and a principle component analysis (PCA) to identify variation amongst replicate chips in individual experimental group. These are shown for my chip data in Figs 6.2 and 6.3

Figure 6.2 shows the signal recorded for each individual control for all nine individual chips, showing very similar responses on all chips, with the same lowest signal (indicating assay sensitivity) as well as the same increase in signal, (reflecting the increase in the concentration of controls). This provided reassurance that (i)

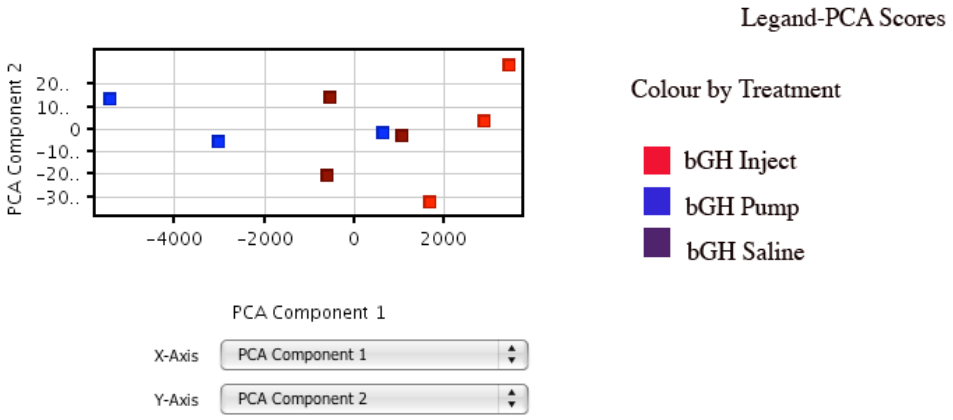
hybridization to the chip was of high quality, and (ii) there was low variation in hybridization efficiency between chips. Figure 6.2 shows a screen shot of the PCA plot for all nine individual chips as separate data points. A color code identifies the two separate treatment and saline (control) groups. The data points were mathematically rotated in relation to one another to maximize the visibility of variability amongst the three separate groups. An ideal result in this display would show the clustering of replicates (colors) in individual treatment groups. Figure 6.2 shows good clustering of individual data points in the groups treated with bGH injection or saline. The clustering for the data points in the continuous bGH treatment group is less obvious but still present. This may suggest a difference in the quality of samples in this dataset and not necessarily a true biological variation within the group and is thus a caveat. Nevertheless the consistency of these quality control tests was encouraging, and allowed me to proceed with analysis.

Figure (6.2) Hybridization controls plot



The X-axis in this graph represents the controls used and the Y-axis, the log of the normalized signal values.

Figure (6.3) Principle Component Analysis (PCA) plot

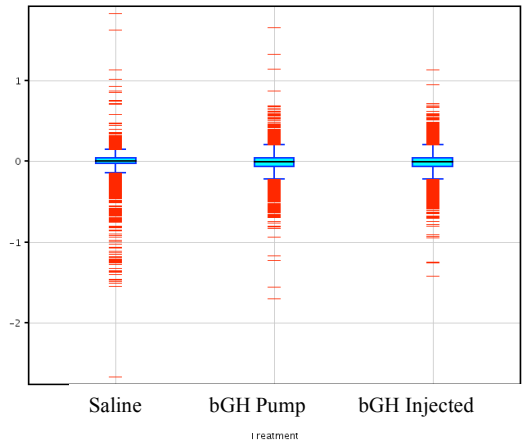


The PCA components are numbered 1 and 2 according to their decreasing significance and can be interchanged accordingly between the X and Y-axis.

6.2.2 Microarray data analysis

The rat 230 2.0 array used contains oligonucleotide probe sets corresponding to 95% of the rat genome. Figure 6.5 shows the distribution of the intensity of values of the probe sets within the three experimental groups (each group consists of 3 replicate chips), following normalization of data using PLIER. In this plot, normalized values of 0 represent a baseline level of probe set intensity values, values greater than 0 represent up-regulated probe sets, and values less then 0 represent down-regulated probe sets. The distribution of values was relatively tight indicating that the level of up regulated and down regulated genes was not vastly different between chip replicates. It is apparent that the group treated with saline shows values to be more widely dispersed when compared to other treatment groups, implying that some individual replicates in this treatment group are numerically more distant from the majority of data, in other groups. Again overall, this data suggests no major inconsistencies in the data sets.

Figure (6.5) Box Whisker Plot for probe set values for individual treatment Groups



The normalized and baseline transformed data are all centered around zero. The Y-axis represents normalized intensity values, X-axis shows each treatment group, which consists of three replica chips.

6.2.3 Filtering Data

All microarray data sets provide huge amounts of data, and generally require filtering. I employed several criteria to generate lists of genes that appeared to be down or as up regulated, and I was also interested to observe which genes had changed expression differentially between the two GH treatment patterns. I first filtered the data to remove probe sets representing genes not expressed in any of my samples, using GeneSpring GX9.0. I chose an arbitrary threshold for the intensity of values obtained for each probe set at the 20^{th} percentile. Values below this were considered as low intensity signals and largely (but not completely) represented genes that I thus "defined" as not significantly expressed. The importance of removing these signals was to minimize the occurrence of false positives, but I am aware that low abundance transcripts could be missed. Larger significant changes in gene expression between groups were then identified using a one-way ANOVA. To assess the magnitude of change, a fold change analysis was also carried out on the probe set values, identifying differentially expressed genes, with a minimum fold change of 1.42. Again, any fold change below this value, was discarded, to reduce data load. Appendix 1 contains all the gene expression values from the three experimental groups.

Strikingly, following comparative statistical analysis of gene expression values amongst the various groups, none of the GH-target genes identified in hepatic array studies had expression values that were statistically different in GH treated groups or even in some cases above the threshold fold change value (exact values are given in Appendix 2).

Although somewhat disconcerting for genes like IGF-1 that are known to be expressed in the placenta, it would appear that GH responsive genes in the placenta did not emerge as significant signals that have been described in the liver using this unbiased approach. Because this was unexpected, and I was concerned it could have related to too severe filtering, I nevertheless felt it of interest to see what changes, if any, occurred in the level of expression in some of these genes in the placenta even if only to document "negative" results.

Placental gene expression values for three of these key GH responsive genes, in the three different experimental groups are shown in Figure 6.7 a-b. Figure 6.7a shows that expression of Stat5b was numerically higher in GH injected animals (0.084) compared to saline injected (0.033), this could be expected as pulsatile exposure of GH is documented to accompany Stat5b activation [61]. However, this is was not consistent within the group of GH injected animals. It was interesting that Stat5b gene expression values were lower in animals treated with continuous GH (-0.054) compared to both saline and GH injected groups, but there also was no consistency amongst the group. Figure 6.7b shows gene expression for IGF-1 in the three experimental groups. As discussed in chapter 1, IGF-1 is known as the classic mediator of the effects of GH, in the GH injected animals IGF-1 gene expression shows a steady increase (0.062) compared to saline injected animals (-0.090). In the continuous bGH treated animals there appears to be a huge increase in IGF-1 gene expression in one of the three animals (0.414) compared to both saline and GH injected groups but again it was not consistent in the others. Thus continuous GH

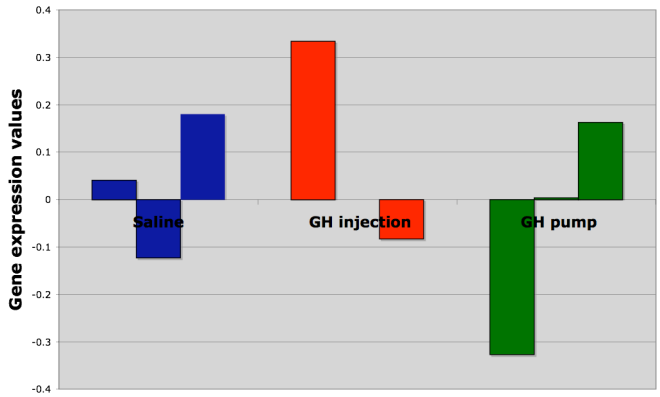
treatment could be effective in activating IGF-1 gene expression in the placenta, but my data does not allow me to draw this as a significant conclusion. This was disappointing, but was only a pilot study it should be repeated as it could be quite an important difference with respect to the liver.

Figure 6.7c shows the gene expression for SOCS3 in the three experimental conditions. The Jak/Stat pathway activated by GH is negatively regulated by the suppressors of cytokine signaling (SOCS)/cytokine-induced suppressors (CIS) proteins [384, 385], CIS proteins have also been implicated in the desensitization of GH-Stat5b signaling by continuous GH levels [386], though the role of SOCS in conditions of continuous GH exposure is less clear. In this figure the most striking observation is the reduced expression of SOCS3 in all three animals injected with GH (-0.013), this implies that pulsatile reduces expression of this gene. In the continuous GH treated group the expressions of SOCS3 is less consistent amongst the animals but show strong up regulation in one (0.124), implying that continuous GH treatment is more effective in up regulating SOCS3 gene expression.

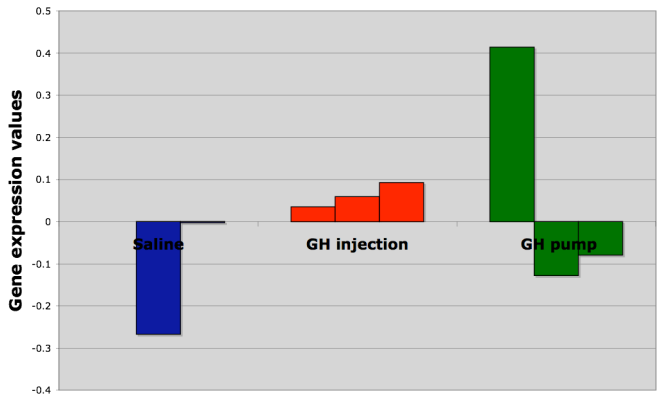
Overall, the pooled data do not generate significant data for these targets, but with such variability, not obviously explained, it is premature to draw the conclusion that these genes are not targets for GH. Perhaps with more numbers of replicates, and attempts to make the experimental paradigms more consistent (or with different time points studied), different results might have emerged

Figure (6.7) Placental gene expression values for (a) Stat5b (b) IGF-1 and (c) SOCS3 in dwarf rats treated with saline, GH injections, or continuous GH (pump) treatment

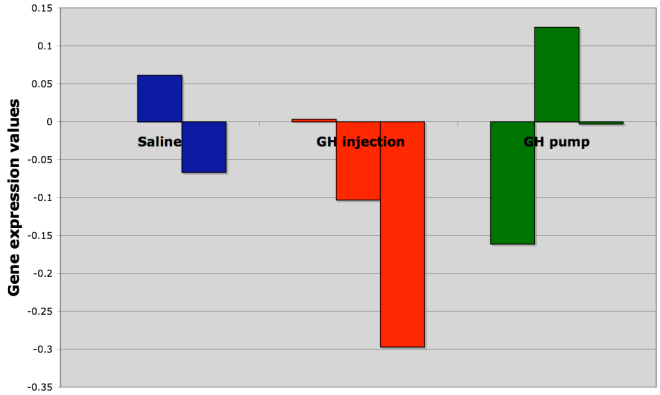
(a)



(b)



(c)



Examination of candidate genes known to be expressed specifically in the placenta, and to contain Stat5b response elements, also showed no statistical differences in expression amongst the treatment groups (expression values can be seen in Appendix 2). This result can of course be affected by a number of technical and fundamental issues, which will be discussed later, but it would appear that this *in silico* approach did not provide me with the hoped for short-cut to experimentally manipulate directly GH responsive genes. Of course the presence of response elements does not automatically imply their use in any tissue, but this approach failed to outline any new GH targets in the placenta. I was therefore left with the third approach, identifying genes that were regulated by GH using an unbiased analysis of expression difference.

All the differentially expressed gene data following the two GH treatments, compared to saline, and to one another, are summarized in Appendix 1. There were 19 differentially expressed genes in the placenta of dwarf rats treated with continuous GH that showed a significant up regulation compared with the saline group. Placental gene expression in dwarf rats treated with GH *injections* showed a significant up regulation of a larger number (55) genes when compared to the saline group. Finally, comparison between the GH injected vs continuous GH treated groups showed 193 genes to be differentially regulated. Genes showing the highest fold changes have been summarized in Tables 6.2-6.4. To attempt to cluster these genes in a meaningful way all genes showing differential expression were then analyzed using Ingenuity pathway

analysis, as this could help identify the canonical pathways that may be enriched in responding genes.

The genes and their categorized functions for all three experimental groups are listed in Tables 6.5-6.7. As often the case, the list contained a mixture of potentially interesting, unknown, and unlikely candidates, and I would have needed to pick and confirm them by qPCR before attempting to speculate on their involvement. There wasn't time to do this at the end of my practical work.

Table (6.2) Differentially expressed genes from rat placenta for analysis based on GH injection vs saline injected (control) rats.

GENE ABBREVIATION, NAME	FUNCTION	FOLD CHANGE
RGD1306603 predicted	Transcribed locus	**2.80**
Dscr1, Down syndrome critical region homolog 1	-	**1.74**
Man1c1, Manosidase	-	**1.67**
Pcdh21, MT-protocadherin	-	**1.65**
Tubb6, Tubulin 6	-	**1.64**
Rn.212222	-	**1.57**
RGD1308967_predicted	-	**1.53**
Grtp1	-	**1.52**
Rn.24461	-	**1.52**
Capn8	-	**1.52**
Rn.205554	-	**1.46**
Fxyd6	Encodes the protein phosphohippolin and is part of a family of 7 FXYD genes (Kadowaki *et al*., 2004)	**1.45**
Klf2_predicted	-	**1.45**
Uhrf1, Ubiquitin-protein like containing PHD and ring finger domain 1.	-	**1.44**
Abcb1a	-	**1.43**
Tagln, Transgelin	Expressed exclusively in smooth muscles.	**1.43**
Bok, BCL2-related ovarian killer	Highly expressed in the ovary.	**1.43**
Rn.146945	-	**1.43**
Atf3, activating transcription factor 3	Member of the mammalian activation transcription factor/cAMP responsive element-binding (CREB) protein family of transcription factors	**1.41**
Slc7a1, solute carrier family 7	-	**1.41**

Table (6.3) Differentially expressed genes from rat placenta for analysis based on GH continuous treated vs saline treated (control) rats.

GENE SYMBOL, NAME	FUNCTION	FOLD CHANGE
Hbe1_predicted, hemoglobin-epsilon locus	Determines the epsilon, or non-alpha, chain of embryonic hemoglobin (originally known as Gower-2)	**2.23**
Hbg1, hemoglobin gamma A	Identified STAT3-like binding sequence in the promoter of gamma-A, (Foley *et al.*,2002)	**1.55**
Transcribed locus	-	**1.52**
Hpgd, hydroxyprostaglandin dehydrogenase 15 (NAD)	Catalyzing the conversion of the 15-hydroxyl group of prostaglandins into a keto group.	**1.46**
Spna1, spectrin alpha 1	-	**1.44**
Prl2b1/ PLP-K, Prolactin family 2 or Prolactin protein-K	A member of the rodent prolactin family.	**1.41**

Table (6.4) Differentially expressed genes from rat placenta for analysis based on GH injection vs GH continuous treated rats.

GENE SYMBOL, NAME	FUNCTION	FOLD CHANGE
Gap43, growth associated protein 43	Crucial component of an effective regenerative response in the nervous system	**4.83**
Arl4a,ADP-ribosylation factor-like 4A	-	**2.71**
Hsd11b2,hydroxysteroid 11-beta dehydrogenase 2	Glycoprotein enzyme-type II, is expressed predominantly in the kidney and placenta and catalyzes only the 11-beta-dehydrogenation reaction	**2.29**
Mllt3,myeloid/lymphoid or mixed	Highly expressed in normal hematopoietic stem cells.	**2.02**
Ampd3,adenosine monophosphate deaminase 3	Plays an important role in the purine nucleotide cycle.	**2.01**
Cuzd1,CUB and zona pellucida-like domains 1	-	**2.01**
Add2,adducin 2 (beta)	Role in assembly of the spectrin-actin lattice that underlies the plasma membrane.	**1.95**
Stag3,stromal antigen 3	High expression in zygotene cells and moderate expression	**1.94**

	in pachytene cells.	
Pklr,pyruvate kinase	PKLR gene encodes pyruvate kinase a glycolytic enzyme that catalyzes the transphosphorylation from phosphoenolpyruvate (PEP) to ADP, yielding pyruvate and ATP. It is the last step of the glycolytic pathway and is essentially irreversible.	**1.92**
Ucp2,uncoupling protein 2 mitochondrial	Glucose metabolism	**1.91**
Hpcal1,hippocalcin-like 1	Hippocalcin-like protein	**1.89**
Amelx,amelogenin X chromosome	Highly conserved proteins secreted by ameloblasts, and constitute 90% of the enamel organic matrix	**1.87**
Gja1,gap junction protein	Expressed in liver, (Li *et al.,* 1995) demonstrated that GAP43-like immunoreactivity in rat is mainly present in sympathetic and sensory nerve fibers as well as in perivascular nerve terminals. This peptide is axonally transported predominantly in sensory and adrenergic axons.	**1.86**
IL10 Interleukin 10	Involved in inhibiting the production of Th1 cytokine production (IFNγ, TNγ, & IL2).	**1.85**
Il18, interleukin 18	Involved in the development of Th1 cells and also in mechanisms of tissue injury in inflammatory reactions	**1.85**
Fxyd6,FXYD domain-containing ion	Encodes the protein phosphohippolin and is part of a family of 7 FXYD genes (Kadowaki *et al.,* 2004)	**1.85**

Ak1,adenylate kinase 1	Muscle development in mice, (Jansson *et al.*,2000)	**1.81**
Prolactin family 2	-	**1.75**
Mpst,mercaptopyruvate sulfurtransferase	Catalyzes the transfer of a sulfur ion from 3-prime-mercaptopyruvate	**1.75**
Nr1d1,nuclear receptor subfamily 1	Provide a direct link between signaling molecules and the transcriptional response	**1.75**
Hbz,hemoglobin-Zeta Locus	Zeta is an early embryonic chain which is substituted for the alpha chain in Hb Portland-1.	**1.75**

Table (6.5) Up regulated placental genes from dwarf rats treated with continuous GH and enriched pathways

GENE DESIGNATION	COMMON NAME	CATEGORY/FUNCTION
HPGD	Hydroxyprostaglandin Dehydrogenase	Cell growth proliferation/ Lipid metabolism
HBE1	Haemoglobin epsilon 1	Organism survival
HBZ	Haemoglobin Zeta chain	Organism survival
BBC3	BCL2 binding component 3	Cell morphology

Table (6.6) Up regulated placental genes in GH injected dwarf rats and enriched pathways

GENE DESIGNATION	COMMON NAME	CATEGORY/FUNCTION
GJA1	Gap junction protein 1	Cell growth and proliferation
KLF2	Kruppel factor 2	"
UHRF1	Ubiquitin-like protein containing PHD and ring finger domains 1.	"
ATF3	Activating transcription factor 3	"
IL18	Interleukin 18	"
MLLT3	Myeloid/lymphoid or mixed lineage leukemia, translocated to 3	"
ADD2	Adducin 2	"
RCAN1	Regulator of calcineurin 1	"

Table (6.7) Up regulated placental genes in continuous GH vs GH injected dwarf rats and enriched pathways

GENE DESIGNATION	COMMONE NAME	CATEGORY/FUNCTION
IL10	Interleukin 10	Molecular transport/canonical pathways
IL-1	Interleukin 1	Canonical pathways
NKαB		Canonical pathways
IL-18	Interleukin 18	Molecular transport/Canonical pathways
NFKBZ	Nuclear factor kappa B	Cell morphology
ADDZ	Adducin 2	Molecular transport
GAP1	Ras p21 protein activator 3	Molecular transport
GAP43	Growth associated protein 43	Cell morphology
HBE1	Haemoglobin epsilon 1	Organism survival
HBZ	Haemoglobin Zeta chain	Organism survival
FYN	FYN tyrosine kinase protooncogene	Cell morphology
DCN	Decorin	Cell morphology
AK1	Adenylate kinase 1	Cell morphology

6.3 Discussion

Oligonucleotide-based microarrays were used in my experiments to attempt to characterize placental gene expression in relation to GH secretory pattern.

Traditional methods of analyzing GH regulated genes in microarrays have mainly employed GH deficient rodent models e.g. hypophysectomized rats, and have identified a number of genes (Table 6.1) as being dependent on GH for expression [381, 387] mainly in the liver. Other GH liver expressed genes include the GH dependent CYP enzymes, which are regulated by GH in a complex fashion, highly dependent on the temporal patterns of plasma GH [48]. DNA microarray analysis has been used to identify genes in rat liver that show sexual dimorphism [388]. Furthermore, microarray analysis has also outlined the important role of Stat5b in regulating the expression of these sex dependent liver genes [389].

Given the time left for these studies, my goal was quite modest, to obtain a preliminary view of the placental gene expression responses to GH, and in particular, to ascertain whether GH, or its pattern of secretion is a major regulatory factor in gene expression in a newly identified GH target tissue. My findings failed to demonstrate that known GH target genes as well as genes containing Stat5b binding sites, known to be expressed in the human and rat placenta were differentially expressed in either GH injected or continuous GH treated animals.

There could be several reasons for this. Assessment of some of the expression values obtained for known (liver) GH target genes in the placenta, although not reaching statistical significance appeared interesting. Stat5b is directly activated in male rat liver in response to each incoming plasma GH pulse, whereas in female rats, the persistence of plasma GH stimulation leads to an apparent partial desensitization of the Stat5b signaling pathway and substantially lower nuclear Stat5b protein, compared to males [61]. What I might have expected was a increase in Stat5b gene expression in animals treated with single injections of GH compared to continuous GH, however, as well as not reaching the threshold set for significant expressional change, the values obtained in my results were also not consistent amongst animals. It could of course simply reflect that the main effect is on Stat phosphorylation, not Stat transcription.

My results do highlight the importance of subject number in experimental groups Although, I showed the placenta to be directly responsive to GH with Stat5 activity (chapter 4), this does not imply that each animal will have the same level of Stat5b gene expression or protein turnover and thus show the same level of response to GH as one another, nor even how effective the Stat5 pathway is in placental cells. A major limitation was my decision to pick a single time point. With hindsight, it might have been better to try to run pairs of treatments at two time points, as I could have missed the peak mRNA changes. For example an experiment, using qPCR to obtain a time course for one target such as IGF-1 might have pinpointed a more optimal time.

As discussed in chapter 1, IGF-1 is best known for mediating the effects of GH, and microarray studies in hypophysectomized rats show GH administration rapidly induces mRNA encoding IGF-1 [387]. Although not significant, my results show a steady increase in IGF-1 levels in GH injected rats, and this expression change tended to be higher in animals treated with continuous GH. In human pregnancy GH is shown to correlate with IGF-1 plasma levels [230] in rodents however, the continuous GH secretion from the pituitary is associated with lower IGF-1 plasma levels [173, 311, 312].

However, it is important to remember that IGF-1 gene expression values shown in the placenta of rats treated with a 48-hour continuous GH pump, are only representative of a single time point following the 48-hour treatment. It is possible that the long-term effects of continuous GH treatment may be reflected in IGF-1 gene expression in later parts of pregnancy. Furthermore, it may also be probable that the continuous GH treatment of pregnant rats may have differentiated effects in different tissues i.e. showing a up regulation of IGF-1 in the placenta, which may have local effects, whilst inducing the down regulating of hepatic IGF-1 gene expression. Unfortunately, since these trends were not significant, in my data, I prefer not to put too much emphasis on this data. Gene expression for SOCS3 was low in the placenta of animals treated with both GH injection and continuous GH treatment. Since Jak2-Stat activity is initiated by a pulsatile GH pattern, a low level of SOC3 gene expression would have been expected in the GH injected animals, with possible high levels of expression in animals treated

with continuous GH. A study by Miquet *et al* (2005) [390] also reports a decrease in liver SOCS3 content in GH over expressing pregnant transgenic mice. These results imply, as in the liver, that SOCS3 may not be directly involved in the desensitization of the Jak-Stat pathway induced by continuous GH exposure in the placenta, at least at a transcriptional level.

Due to the lack of significant change in Stat5 gene expression, I am unable to draw any firm conclusions about regulation of this gene in the placenta. It is possible that gene expression changes expected in response to GH may have been diluted amongst a high level of background, owing to non GH responsive cells in the placenta, unlike in the liver. Genes containing Stat5 response elements may be expressed only in a minority of specific cell types that make up some of the RNA, which could have contributed to clearer observation of small changes in gene expression. However, observing placental gene expression in isolated cell types may not be physiologically representative of what happens in the placenta. Furthermore, there could also be negative implications in gene expression when isolating cells from other near by cell types. Whatever the reasons, this approach via *in silico* "targeting" of Stat5 potential regulated genes did not provide me with the short cut to Stat5 regulated targets in my study.

Although many sex-dependent liver genes are regulated by sex differences in pituitary GH secretion [301] such roles of GH secretory patterns in placental gene expression have never been shown, and although I have shown the pYStat5 response in the placenta to be sensitive to GH secretory pattern, this may not necessarily be

implicated in the expression of the same genes known to be pattern sensitive in the liver. It is also important to note that only one time point after both GH injected and continuous GH treatment was assessed in the placenta. It is quite possible that this specific time point may have not been adequate in observing the higher end of transcriptional changes in the placenta, since GH and IGF-1 levels progressively change during both human and rodent pregnancy. Finally, because in my studies, I identified GH response in the placenta by visualization of Stat5 activity, I hoped that genes with Stat5b response elements might show changes upon GH administration. However, its important to consider in this new GH target tissue, that there may be several other GH activated signal transduction pathways. Therefore, a number of genes transcriptionally responsive to GH, that emerged in the unbiased approach may not be directly regulated by Stat5 or indirectly regulated by other transcription factors, or even affected by other pathways such as AKT or MAPK. Thus, I was left with analyzing the genes that did show statistically significant changes in response to GH, from the unbiased approach.

Administration of GH in the two secretory patterns did up-regulate several genes, of which some responded in common to both secretory patterns. The majority of these genes have unknown functions. All of the differentially expressed genes were also analyzed using enrichment pathway analysis, which resulted in some of the genes being categorized in several canonical pathways, as well as functions. However, possible involvement in growth or metabolism remain unclear due to a lack of experimental documentation, and whilst some are interesting (e.g. IL-10) others are

likely not to be very relevant (e.g. Hippocalcin-like protein). In all cases, to take this further I would need to confirm those changes independently in replicate experiments and by qPCR, and before attempting this it would be further beneficial to first check different time points. This would be a major study.

My results do show that the Hydroxyprostaglandin Dehydrogenase (HPGD) gene is statistically up regulated in animals treated with continuous GH treatment compared to saline. The HPGD gene is expressed in the human placenta and its main function is to regulate contractility of the myometrium [391]. This gene has also been correlated with cases of pre-eclampsia, in which gene expression is reduced [392] if this is confirmed it might imply a potential role of continuous GH secretion in the regulation of a genes important for the progression of pregnancy to term. Also in the continuous GH treated group, there was a significant fold change in prolactin protein K (PLP-K). Rat PLP-K is part of the rodent prolactin family [393]. PLP-K expression is restricted to trophoblast cells within the labyrinth zone, expression has been reported to be initiated in trophoblast giant cells at midgestation and then extended to spongiotrophoblast and labyrinthine trophoblast cell types as gestation progressed [394] however, the nature of its physiological actions remain unclear.

Interestingly, a member of the interleukin family is up regulated in animals treated with GH injections, compared to animals treated with continuous GH. Interleukin 10 (IL-10) is involvement in the regulation of Jak-Stat signaling pathway [395] and has the potential role of activating other cytokine initiated pathways independent of Stat5. Stat3 was originally discovered as a factor activated by IL-6 through gp130

receptor [396]. However, Stat3 is also documented as being essential for the actions of IL-10 [397]. The importance of Stat3 in embryonic development is emphasized with the deletion of Stat3, resulting in embryonic lethality due to placental insufficiency in leukemia inhibitor factor (LIF) signaling [398]. Mice in which Stat3 is deleted in a tissue and cell specific manner have been generated [398]. These mice have revealed critical roles of Stat3 in liver acute phase responses [399]. A study by Cui *et al* (2004) [400] also shows that the loss of STAT3 in the hypothalamus caused by RIP-Cre action interfered with normal body weight homeostasis and glucose metabolism. Thus, GH secretory pattern may be important in initiating other signal transduction pathways, independent from Stat5, which may be of great importance in the survival of a fetus from an early embryonic stage. Again, such speculation requires further confirmation of changes.

In summary, a preliminary study to identify potential GH target genes in the placenta was justified on the basis that the placenta was (a) responsive to GH and (b) expressed genes known to contain Stat5b response elements. However, despite no known GH targets or genes containing Stat5b response elements showed statistical expressional change in response to GH or a change in secretory pattern, new genes of which a few, pose as potential candidates that could have some importance in growth during pregnancy. These results act as preliminary data that could prove useful if future, more thorough studies were to be performed.

General discussion & Summary

7. General discussion

I have discussed the detailed results in each chapter, so will finish with more of an overview of the work I accomplished. The characterization of a human growth hormone variant (GH-V) [227] whose sole source of production is the placenta [202] has provided another endocrine source of GH for systemic actions in the mother. However, evidence that the human GH receptor is also expressed in the placenta [229] raises the interesting question of whether the placenta itself could be a direct autocrine or paracrine target of growth hormone, as it is well recognized as a complex endocrine organ that mediates a number of events in adaption of maternal tissues to pregnancy. GH in the placenta could be acting constituting in an autocrine or paracrine fashion, and subject to regulation. Despite much circumstantial evidence for association between GH levels and various functions most of these were not related to placenta as a target. Thus all the evidence for this before I started my thesis work was indirect.

My primary aim was to establish at a cellular and tissue level, whether there were cells directly responsive to GH in the placenta. The results I obtained were generated from both normal and rodent models of GH deficiency, characterized by others when I joined the Robinson lab. The advantage of using these well-characterized models of GH deficiency was that I was able to observe the direct effects of administered GH without the interference of existing GH. This also avoided creating conditions of excess GH, which would have otherwise lead to the problem of not being able to clearly define the effects of the GH being administered from the effects caused by a more pathological condition created with an excess of GH. Nevertheless it was important to me to confirm that results I obtained in these GHD models would be

reproduced in principle, in intact models. Using GH deficient rodent models gave me the possibility to vary the pattern of GH administered, to assess the changes in cellular response with the changes in GH secretion pattern in the placenta. Finally, using both rat and mouse models of GH deficiency enabled me to assess more than one rodent species.

An obvious question is how relevant my observations in rodent pregnancy may be to humans. There is no ideal model for human pregnancy other than humans, however mice and rats have the practical advantage of having short generation times and large litters. Indeed, in choosing appropriate models for human pregnancy, one criteria might be how close they are to humans from a phylogenetic standpoint, the mouse appears to be reasonably close to primates, this is strongly supported by molecular phylogenetics reviewed by Springer *et al* (2005) [401], though this has been questioned by reports of unusually high rates of mutation [402]. Analogous cell types have been identified among human and rodent placenta, particularly in the trophoblastic linage including proliferative trophoblastic cells, invasive trophoblastic cells and cells differentiating into syncytium [274, 275, 403, 404].

One of the major differences in the human and rodent placentas lies in the difference in their endocrine function. In mouse, progesterone production by the corpus luteum is required throughout gestation [405] this is regulated in the first 8-9 days by mouse pituitary prolactin [406]. From around day 11 of gestation, mouse placental lactogen produced by the trophoblastic giant cells take over from pituitary production of prolactin. The lack of mouse pituitary requirement during late pregnancy is supported by studies that show the successful progression of pregnancy following

hypophysectomy in mice performed after day 11 [405]. In humans also, the pituitary gland is not required for the initiation or maintenance of pregnancy. Maintenance of the corpus luteum depends initially on hCG production by trophoblast cells, and after 8 weeks of gestation, placental progesterone production by the syncytiotrophoblast cells is sufficient to maintain pregnancy even in the absence of ovaries [407, 408]. A more important difference for me, in human and rodent placental endocrine function, is the lack of any placental source of GH in rodent pregnancy. Such endocrine differences between rodent and human placenta may reflect the great fundamental difference in gestation length and the birth of altricial young in rodents. Many of the developmental processes that occur in humans during intrauterine life are postnatal events in rats and mice, so these timings are not strictly comparable.

Taking into consideration the absence of a placental source of GH and the large production of placental lactogen by the rodent placenta, it is difficult to outline the possible functions of GH during rodent pregnancy. However, there is evidence that has shown the presence of GH receptors in both fetal and maternal compartments of the rat placenta [332] providing circumstantial evidence for the idea that the rodent placenta could be a direct target for GH. Furthermore, although the somatomedin hypothesis [85] favors the notion that most actions of GH is classically thought to be mediated via IGF-1, it is now recognized that GH has direct actions via the GH receptor, in a number of tissues [409]. When I began my thesis, work what was missing was a direct demonstration of GH activity. Since Gevers *et al* in the lab had produced a method that was suitable to the task, my main aim was to use this to obtain the direct evidence for a direct GH action in the placenta. My results showed this is indeed the case.

The secretion pattern of pituitary GH in pregnant rodents has been shown in several studies [81, 225, 226] to mimic the secretion pattern of GH-V in human pregnancy. The identification of GH receptors in the rat placenta and the documentation of such a GH secretory pattern change provided some evidence to suggest that the rodent placenta may act as a direct target of GH and be potentially involved with indirect mechanisms that may be favored by a more continuous pattern of GH. Thus, using a rodent GHD model with GH replaced in different patterns would be appropriate to shed some light on the possible functions of GH-V during both rodent and also human pregnancy and more importantly the possible importance of the GH secretory pattern change.

At the start of my thesis plan, I wished to see if I could obtain preliminary evidence for this in human placental cells *in vitro*. This would bridge the species gap and perhaps led to early optimization of GH responses. Unfortunately, I was unable to detect any endogenous GH or GHR in either of the placental cell lines used, despite previous documentation [282]. Furthermore, although the placental cell lines were shown previously to be transfectable [292], my attempts to supply the cell lines with components of the GH signaling cascade (GHR and Stat5b constructs) resulted in minimal cell survival and inability of few surviving to respond to exogenous GH administration. These results proved likely to be a lack of appropriately differentiated cell type, since I readily obtained GH responses in Hep2 cells with the same method.
A major frustration was that my original hope for *in vivo* studies was to use a mouse transgenic line already reported to produce an autocrine source of hGH in the placenta [410]. In hindsight, it would have been quicker to re-make the CYP19-hGH mouse, rather then wait for it to arrive (there were numerous difficulties in the Mendelson

lab), but by the time it was evident that this mouse model was not going to arrive in time for me, I decided it was too late to make my own transgenic. This would still be an interesting model to pursue, perhaps also using GH antagonist [411] to interrupt endogenous signaling in this mouse.

In order to define responses to GH I wanted to exploit a method that would unequivocally identify activation of the GH signaling pathway. Using immunohistochemistry I was able to directly reveal individual nuclei that responded with Stat5b phosphorylation and nuclear translocation to a single acute injection of GH. This was shown for the first time in both the rat and mouse placenta. The reliability of this method is supported with previous studies, which have used this experimental approach to identify GH responding cells in a variety of other tissues (e.g. cartilage, liver, fat, heart, and muscle) [291, 412]. It has proved better than Jak2 or other kinase markers (Gevers, personal communication). The response shown in the placenta following bGH is likely to be via the GHR. In rodents bGH binds only to GH receptors, while the use of human GH would show binding to both GH and PRL receptors [413]. This study by Kopchick showed that all analogues of bGH show ability to bind to GH receptors and exhibit somatogenic activity *in vitro* and *in vivo*. However, none of these bGH analogues show binding to PRL receptors or elicit detectable lactogenic response. However, there are a number of things that should be kept in mind when considering immunostaining of phosphoStat5 in GH signaling in the placenta. Firstly, this method only identifies Stat5 responses in cells, this doesn't imply that the remaining cells not showing Stat5 phosphorylation are unresponsive to GH. There could be a number of pathways in the placenta that are activated by GH e.g. MAPK/ERK pathways, so several other methods would be needed to completely

dissect and outline the full repertoire of potential GH activated pathways in the placenta. Second, tissues were obtained only on day 16 of gestation, this day was chosen as it's the time point in which GH levels peak in normal rodents [62]. It was therefore considered a time point in which tissues could be expected to be most sensitive to GH, thus chosen as the day to administer GH. However, day 16 of pregnancy may not be the peak time of phosphoStat5 responses. Nevertheless, a clear marked activation of Stat5 was shown in both the placenta and the positive control (liver) compared to saline injected control animals. Again, other cells could be responding at different times, and in future studies it could be interesting to determine when the placenta first shows GH responses.

The level of response in all tissue sections was assessed using a novel quantification method. My analytical approach identified individual responding nuclei as well as calculating the level of response by individual staining intensity. As discussed earlier, all semi-automated methods tend to involve some compromise in settings. For example the thresholds set to exclude any non-specific or background staining may have been marginally too stringent or wide, allowing the discarding of some responding cells, or creating some false positives. There were large differences in background and non-specific staining observed in some individual animals, therefore making it very difficult to apply arbitrary margins to all individual tissue sections. Despite these limitations the analysis showed significant difference between GH and the different doses administered, compared to saline injected animals, and was much more practical than subjective visual analysis of all the sections.

To further demonstrate the direct action of GH, it was considered important to show the expression of GH receptors in the rat and mouse placenta. Although this has already been reported in rodents [414] and humans [229] I was unable to show any reproducible localized GHR expression in either the rat or mouse placenta using my

reagents. The antibody used has been designed to recognize the extracellular part of the human, rabbit, and rat GH receptor [22]. However, my protocol was probably not sensitive enough, particularly in cases of low GHR expression. In the case of the placenta there is no evidence to suggest the levels of GHR expression are higher or lower, compared to other tissues, but I believe that there was too little GHR expression to be detected by this antibody and method in hand. Previous attempts to show GHR expression in other mouse tissues has been quite difficult, though data has been successfully reported for the growth plate [291]. The ability to detect antigen is dependent on the protein digestion step used in the immunhistochemistry protocol, as well as the method adopted to fix the harvested tissue. Despite several attempts to optimize these steps for placenta no improvement was observed in GHR staining.

The effects of GH on the expression of many hepatic GH-dependent genes are known to be highly dependent on the pattern of exposure for the stimulation of growth in rodents [50, 362] and humans [415]. In rodents, this has been directly linked to the role of Stat5b, as deletion of the gene has resulted in compromised pattern of male-specific growth and expression of genes responding to pulses of GH [36]. In female rodents, GH secretion pattern is more continuous, this continuous GH secretory pattern is more pronounced during both rodent [81] and human pregnancy [219, 220], and generates different patterns of gene expression [37, 61]. I therefore decided to look at the effects of continuous GH exposure on Stat5b *per se*, and how such pre-exposure might alter the effects of a GH pulse. Given that in pregnancy, the predominant GH pattern is continuous, continuous GH infusion did increase the number of nuclei exhibiting phosphorylated stat5b under basal conditions, in both liver and placenta from rats and mice, but it tended to desensitize the system as there was very little difference when animals were given a further injection saline or GH. Overall, however, my results support previous studies that have used GH deficient rodents models to demonstrate the differential effects of GH on Stat5b [78, 362, 416] as well as confirms previous studies, which have also shown that continuous GH secretion blunts the acute effects of GH on

pYStat5 signaling [291, 412]. Although these findings demonstrate that continuous pattern of GH exposure can still evoke GH initiated Stat5b phophosphorylation in the placenta, its quite clear that this pattern of GH exposure is not best at revealing an acute effect of GH on pYStat5. Again it is important to recall that Stat5b mediated pathway may not be the only GH initiated pathway taking place in the placenta, as in other tissues [39]. It is possible that GH-GHR activation of Stat5b may be involved in activating more downstream targets that are then implicated in pregnancy, and of course IGF-1 will have Stat5 independent activities, with the known relationship between continuous GH secretion and circulating IGF-1. Continuous increasing GH exposure could alter the balance between direct and indirect (IGF-1 mediated) GH actions during pregnancy, and desensitize the tissue to acute changes in GH signaling. My result does not rule out the possibility that GH can also act indirectly (e.g. via IGF-1) on the placenta.

In humans this could be via local paracrine activation of placental IGF-1, or via the circulation to elevate hepatic production. The pattern of GH secretion can also be important in the regulation of GH receptor expression itself in different types of tissues, which in turn, can regulate GH initiated pathways involved in pregnancy. This is supported with studies by Iida *et al* (2004) [417] which have shown that excess GH in transgenic mice leads to increased levels of GH receptor expression in the liver but a decrease in expression levels in muscle tissue. However, no study has yet to report changes in GH receptor expression in the rodent placenta during pregnancy, and my results suggest this could be worth investigating, once GHR can be reliably detected. GHR expression and signaling is known to be reduced in fasting animals [80, 349] my findings also confirmed reduction in GH signaling via pYStat5 in both the liver and the placenta of animals fasted for 48 hours.

Remarkably, a single acute injection of insulin some hours prior to GH was also shown to restore a significant level of GH signaling in both the liver and placenta, even whilst fasting continued. I suggest that this is a direct effect of insulin independent of nutritional uptake, and suggest that signaling cascades may be able to integrate with the GH signaling pathway, thus potentially regulating metabolic activity of the cell. GH has been shown to regulate the IRS cascade directly via IGF-1 and also act indirectly on insulin signaling, [418]. This crosstalk of the GH and insulin signaling pathways, if it occurs in placenta, may be important in regulating an important balance between growth and metabolic responses. My results suggest the crosstalk with the insulin signaling pathway may be more important in cases of low GH, in which it could activate the metabolic reserve of the cell, enhance to the Stat5 signaling cascade and GH action. The cross over of the two pathways could also be debated in cases of excess GH, since insulin resistance is a hallmark of Type 2 diabetes in which high GH levels are ineffective. The importance of such a balance between the two signaling pathways could be implicated in cases of gestational diabetes, and the potential role of GH-V thus warrants further investigation [419, 420].

Finally, what might be the physiological role of placental actions of GH? Although I have shown the placenta to be a direct target of GH and sensitive to GH secretory pattern, I haven't attempted to show any downstream effects, and how GH is implicated in pregnancy functionally is far from understood in any species. My data in GH deficient models reveal some differences in the outcome of pregnancy in the absence of GH, when compared to normal rodent pregnancy, particularly in mice with a significant reduction in the number of pups born to each litter in both dwarfs and GRF-M2 mice. It is not clear how this would be directly caused by a lack of GH. It may be more likely to be the physiological deficits within the mother as a result of the absence of GH, but further experimental approaches will be needed to explore this further in animals. Contrasting findings in my results were seen in the individual pup weight data. Pups born to dwarfs showed similar weights to pups born to normal rats, however, pups born

to GRF-M2 mice showed a significant reduction in weight, compared to pups born from normal mice. It is well documented however that the direct actions of GH are not needed for fetal growth, as Laron dwarfs that have no functional GH receptors are born with normal weights [99]. Therefore, the reduction observed in pup weights could more likely reflect effects in the mother, affected by the GH deficiency, resulting in varied physiological changes that may then indirectly affect the growth of the individual pup, from complex metabolic stages to the more mundane restriction of smaller uterus [315, 421-423].

One direction for future work lies in looking at the possible targets of GH in the placenta. I only had time to perform preliminary experiments on this, but my initial data from microarray analysis carried out on placenta treated with continuous or pulsatilic GH points to some genes that may be targets of GH in the placenta, and that may also be sensitive to the pattern of GH. Obviously, until now, few studies have directly looked at changes in gene expression in the placenta in response to GH, most studies have only concentrated on using knockout mice to identify genes involved in placental development, or to look at GH responses in other target tissues such as liver, bone, muscle and fat [87, 133, 275, 424]. Given the shortness of time, I entertained two quick short cut approaches to attempt to identify potential new GH target genes in the placenta. The first was to check whether there were any changes in known GH targets genes [381] and the second was to look at any changes in genes expressed highly in the placenta [425] identified to contain Stat5b response elements from my *in silico* searches.

Unfortunately none of the genes from these predicted lists were found to show any expression changes in any of the groups. This was unexpected but given some of the technical limitations of the experimental setup, I concluded that this limited approach was not ideal for studying more obvious GH target genes, a particular area to consider would be the isolation of specific cell types in the placenta, to be able to observe smaller changes in particular genes, and to go directly for qPCR analysis. This has been feasible

in the study by Sood *et al* (2006) [425], who isolated the various tissue types that make up the placenta. I therefore fell back on an unbiased approach by looking at the most up-regulated genes. Here I was able to identify few genes which could potentially have GH initiated roles in the placenta, however, these findings would initially need to be confirmed by repeat array studies, qPCR and western blot analysis, and was beyond the scope of my thesis work. The opportunity for further work would also arise from mouse models, specially expressing GH in the placenta [410]. The effects of having a placental source of GH could be then assessed and compared to normal rodents, which only have the pituitary source. Furthermore, comparative studies in these models could also be approached to look at differential placental gene expression in rats vs mice. Possible candidates would hopefully identify other GH initiated signaling pathways [29] that might also be present in the placenta and coupled to GH.

In conclusion the findings of this thesis provides evidence for the first time that the placenta itself is responsive to GH, and as in the liver the activity of Stat5 in response to GH remains sensitive to pattern change and fasting. GH signaling in the placenta is clearly interactive with other pathways, however, the function relevance remains to be determined. It is evident that GH-V during human pregnancy remains important for the regulation of systemic IGF-1, an important mediator of the effects of GH, however, where these actions takes place remains unclear. My work suggests that the placenta itself could be a direct target for GH-N in rodents, and also an autocrine or paracrine target for this placental hormone in humans.

Appendix 1
Microarray data for all experimental groups

Condition (1)*S	Saline	Saline	Saline	bGH pump	bGH pump	bGH pump	bGH inject	bGH inject	bGH inject		
Replicate (1)*S	1	2	3	1	2	3	1	2	3		
SystematicFold chan	Flags	Raw Flags	Raw Flags	Raw Flags	Raw Flags	Raw Flags	Raw Flags	Raw Flags	Raw Flags	Raw	Common Gene Sym	Description
1379640_ 2.713	P	10.77992 P	13.85063 P	8.690509 A	3.702056 A	3.703733 A	4.79545 A	5.430086 A	7.174631 A	7.021972	RGD:73511	ca69-related protein
1376702_ 2.557	M	9.659393 P	12.81154 A	10.06985 A	3.411409 A	6.752596 A	3.276613 A	6.879062 P	7.728528 A	11.72254		Megalencephalic leukoencephalopathy with subcortical cysts 1 (predicted)
1396169_ 2.53	P	13.62259 P	14.76814 P	12.01872 A	5.545246 A	6.154826 A	4.427285 A	12.63413 P	17.03307 P	16.16288		Similar to 4921510J17Rik protein (predicted)
1391860_ 1.952	P	12.15526 P	8.310773 A	13.29561 P	6.790933 A	4.187118 P	6.42303 P	6.929717 P	12.8683 P	14.33306		Transcribed locus
1392749_ 1.947	P	24.38401 A	16.20874 A	20.49417 A	9.632067 A	9.431266 P	12.21927 A	16.53331 A	14.08324 A	26.8714	RGD:1303	ciliary neurotrophic factor receptor
1385445_ 1.906	P	10.7024 A	13.15372 P	13.39647 P	7.732199 P	4.600934 P	7.748235 P	15.52205 P	8.989033 A	5.735563		Similar to MGC19222
1385411_ 1.903	P	29.67041 P	28.42032 P	26.94514 P	12.84641 P	17.23586 P	15.0664 P	19.36553 P	17.21324 P	33.27647	Usp43_pr	ubiquitin specific protease 43 (predicted)
1383691_ 1.876	P	6.566653 P	10.80156 P	6.937471 A	3.595199 A	3.664352 P	5.721756 P	4.550557 A	4.269024 A	3.084973	Atf2	Activating transcription factor 2
1391648_ 1.874	A	6.023252 A	7.570943 A	10.1589 A	3.578971 A	3.613199 P	5.509497 A	5.447877 M	9.114609 P	4.5254		Transcribed locus
1380464_ 1.83	P	11.28954 P	11.44844 P	16.91276 A	6.509322 P	7.288511 P	7.607799 P	10.54762 P	16.03762 P	6.50339		DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26 (predicted)
1381615_ 1.795	P	17.47716 P	12.02107 P	16.18766 P	9.712169 P	9.983653 A	6.132986 P	12.18366 P	9.994355 P	11.68631		Transcribed locus
1380172_ 1.785	P	26.11014 P	35.75691 M	26.47273 P	16.84911 P	15.36455 P	17.00222 P	14.78975 P	30.7862 P	25.06493	Kif5c_pre	kinesin family member 5C (predicted)
1394666_ 1.784	A	15.16811 M	17.83056 A	11.29187 A	10.01751 A	6.816103 A	7.969485 A	10.21088 A	14.76433 P	9.770338		Nebulin (predicted)
1385010_ 1.768	M	25.53793 M	29.2675 A	23.98879 A	11.46529 A	15.84626 A	18.07081 A	20.49326 A	12.86924 P	24.47604	RGD13062	similar to RIKEN cDNA 2310015J09 (predicted) ; type I hair keratin KA28
1378636_ 1.763	P	16.98253 P	22.12777 P	17.55281 A	12.90274 P	11.17567 A	8.450365 P	17.28775 P	17.96291 A	9.276158		
1396274_ 1.759	A	17.45021 P	14.90578 P	10.81653 A	9.02271 A	7.249119 M	7.997824 A	20.87442 A	10.82764 A	12.41279		Transcribed locus
1380870_ 1.711	P	8.630274 P	7.536034 P	5.637979 M	4.541284 A	4.230956 A	3.852777 P	7.446205 A	2.509029 P	5.292166		Similar to RIKEN cDNA 1700040L02 (predicted)
1368236_ 1.709	P	10.07741 P	13.08116 P	13.28805 A	8.018105 A	7.520377 A	5.885786 P	14.59264 A	6.65133 A	10.99603	Mep1a	meprin 1 alpha
1383537_ 1.708	P	22.51845 P	16.66366 P	15.32966 P	9.76761 P	11.79727 P	10.13645 P	10.77893 A	9.316725 P	22.11298		Vascular endothelial zinc finger 1 (predicted)
1378197_ 1.694	P	26.18667 P	22.04566 P	28.64405 P	16.98094 P	15.33379 P	13.21016 P	22.70922 P	25.93511 P	16.80478	KIFC2	kinesin family member C2
1372868_ 1.692	A	40.87006 A	33.80103 P	41.41739 A	29.61733 A	19.11258 A	21.11753 P	27.39152 A	29.14037 A	33.72636	Tor3a_pre	torsin family 3, member A (predicted)
1384680_ 1.678	P	49.20684 P	38.85344 P	49.33643 P	26.98219 P	30.02099 P	24.95429 P	55.41842 P	45.12899 P	32.21533	Mtus1	Mitochondrial tumor suppressor 1
1379577_ 1.672	P	15.57579 A	11.61141 A	15.13466 A	8.370804 A	6.643561 M	10.65177 P	14.42327 P	11.08773 P	13.72799	LOC50041	similar to MIC2L1
1377305_ 1.67	P	16.56577 P	17.45681 P	25.14481 A	12.61299 P	10.08331 A	12.42522 A	15.72174 P	11.05621 A	10.98244		Hypothetical LOC310764 (predicted)
1382186_ 1.666	P	17.40069 P	21.23754 P	15.95962 P	10.16471 P	10.96632 P	11.586 P	14.00376 P	14.44578 P	17.06826	RGD1311	similar to RIKEN cDNA 2610029K21 (predicted)
1377337_ 1.644	P	7.107294 M	9.805884 P	9.886682 P	5.680533 A	4.193733 A	6.588985 A	7.080904 A	8.175183 P	12.41756		C-myc binding protein (predicted)
1384609_ 1.64	P	28.13092 P	22.53942 A	33.8396 P	16.68381 A	14.24093 M	20.71094 A	20.91782 P	19.57934 P	20.36471	RGD1311	similar to RIKEN cDNA B230380D07 (predicted)
1396391_ 1.618	P	16.6269 P	12.77971 A	13.88224 A	10.42817 A	9.563114 A	7.069953 P	11.65766 P	18.06944 A	16.95234		Similar to male sterility domain containing 2
1370816_ 1.611	P	20.68779 P	18.25483 P	24.7642 A	12.22181 P	12.812 P	14.44397 P	36.452 P	28.80547 P	24.74142	Nr1d1	nuclear receptor subfamily 1, group D, member 1
1380964_ 1.598	P	7.578683 P	9.126699 A	6.99791 P	4.551583 A	5.152765 A	5.114914 A	4.458827 A	3.124033 A	11.02285		Similar to dystrobrevin alpha isoform 1
1375386_ 1.575	A	7.742449 P	10.12062 P	11.03664 A	5.204338 P	7.421891 A	5.798077 A	0.73189 P	8.76455 P	7.712946		Similar to mKIAA0804 protein (predicted)
1396280_ 1.571	P	97.26366 P	82.02874 P	86.26887 P	57.73859 P	55.19388 P	56.38005 P	66.32711 P	56.99611 P	58.2047	LOC30256	similar to G patch domain and KOW motifs
1396945_ 1.564	P	13.03185 P	13.25448 P	10.16522 P	9.54638 P	6.2849 P	7.745923 A	0.414169 P	5.04770 P	8.052024		Transcribed locus
1397025_ 1.562	P	35.96549 P	44.74784 P	49.55952 P	28.5283 P	29.60945 P	25.09863 P	22.75339 P	43.02403 P	30.51071	Trpc4	Transient receptor potential cation channel, subfamily C, member 4
1392561_ 1.561	P	17.67604 P	17.1522 P	19.07759 A	13.33744 A	11.63529 A	9.912245 A	11.31089 A	10.77174 P	12.86178	Taf3_pred	TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor (predicted)
1385332_ 1.561	P	13.89664 A	12.77755 P	16.83784 A	8.179609 A	10.4486 A	9.310978 A	12.14819 A	7.386911 P	9.679131		Similar to protein similar to E.coli yhdg and R. capsulatus nifR3 (predicted)
1398688_ 1.555	P	25.76149 P	36.00822 P	35.50203 P	17.95619 P	18.9164 P	26.0994 P	32.2333 P	32.03444 P	33.1511	Spink5_pr	serine protease inhibitor, Kazal type 5 (predicted) ; similar to Spink5 protein
1396518_ 1.547	A	6.772033 P	8.620581 P	9.400674 A	4.581282 A	4.953662 P	6.6042 M	6.183833 A	5.288638 M	9.64873		FYVE, RhoGEF and PH domain containing 3 (predicted)
1376781_ 1.537	P	120.1821 P	120.5277 P	121.3185 P	75.50466 P	83.43182 P	77.79057 P	86.84746 P	118.9824 P	103.8944	Glb1	Galactosidase, beta 1
1391684_ 1.534	A	31.45784 A	28.27786 A	33.71306 A	18.70006 A	24.60668 M	18.26015 A	24.37549 P	31.73697 A	32.52305	Tmem14a	transmembrane protein 14A (predicted)
1385692_ 1.529	A	47.09114 A	38.35324 M	40.15428 A	28.75455 A	23.95456 A	29.823 A	44.95573 M	47.81514 A	39.76297	Slc10a4_	solute carrier family 10 (sodium/bile acid cotransporter family), member 4 (predicted)
1379648_ 1.524	P	16.52596 P	15.92761 P	13.62533 P	9.752743 P	10.0744 P	10.42781 P	13.78924 P	10.93379 P	10.42577	Nfat5	Nuclear factor of activated T-cells 5 (predicted)
1368061_ 1.522	P	16.94209 P	20.80366 P	18.32871 P	11.50674 P	10.58676 P	15.21627 P	17.92921 P	10.62808 P	14.08158	Kcnh1	potassium voltage-gated channel, subfamily H (eag-related), member 1
1380923_ 1.519	P	70.58188 P	62.92563 P	52.47189 P	39.71723 P	38.48633 P	44.03976 P	67.86451 P	62.45996 P	71.59325		N-acylsphingosine amidohydrolase 3-like (predicted)
1368432_ 1.517	M	16.10072 P	13.30107 M	12.03697 P	10.12853 A	7.205982 A	10.23038 P	16.14782 P	8.900972 A	9.56637	Ros1	v-ros UR2 sarcoma virus oncogene homolog 1 (avian)
1398479_ 1.517	P	20.55633 A	16.13204 P	18.42417 P	12.22859 A	12.08684 P	11.99461 P	20.04779 P	16.69371 A	8.694668		Transcribed locus, weakly similar to NP_996757.1 ryanodine receptor type 3 [Gallus gallus]
1381471_ 1.516	P	42.06404 P	38.65163 P	44.8533 P	29.1869 P	30.29579 P	23.93569 P	28.05773 P	33.9259 P	54.83676		Transcribed locus
1391386_ 1.515	P	6.447806 A	6.329239 P	6.214696 A	4.734459 A	3.678133 A	4.241419 P	7.20564 A	6.95028 A	4.965504	Chn1	Chimerin (chimaerin) 1
1367937_ 1.51	P	66.7809 P	67.76386 P	69.31418 P	44.97873 P	42.66479 P	48.00059 P	61.01009 P	69.07107 P	60.81094	Miox	myo-inositol oxygenase
1397154_ 1.502	A	27.68133 P	25.20919 P	22.3262 P	19.77136 P	16.54128 P	14.23299 P	16.30015 P	12.24069 A	24.71881		Myeloid lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)
1370520_ 0.663	P	22.84478 P	19.74659 P	15.89831 P	31.92809 P	23.61837 P	32.97791 P	22.52879 P	16.84199 P	16.9166	RGD:72779	hippyragranin
1376180_ 0.663	P	45.22911 P	35.92415 P	44.92118 P	71.50909 P	51.4601 P	68.80342 P	31.85651 P	40.14759 P	48.5204		
1391571_ 0.658	P	14.45683 P	13.11981 P	11.76632 P	22.26709 P	17.92402 P	19.85229 P	12.26607 P	9.562852 P	12.00377		Transcribed locus
1376403_ 0.658	P	7.899254 A	9.165629 P	9.678021 P	13.5218 A	11.48969 P	16.02577 A	13.36984 P	9.511164 M	13.32318		
1396993_ 0.657	A	22.11608 A	30.64543 A	25.30629 M	39.65807 P	41.91699 A	36.75429 A	17.3666 A	33.30009 P	36.21574		Transcribed locus
1390950_ 0.657	A	6.335732 A	7.173213 P	5.00602 P	9.836214 A	8.502183 P	9.696831 A	4.977945 P	7.558811 A	5.457897	Prdx3	Peroxiredoxin 3
1383384_ 0.654	P	58.72894 P	75.73902 P	61.86374 P	80.20663 P	111.8061 P	110.8859 P	81.34619 P	87.02633 P	73.55166		TATA box binding protein-like 1 (predicted)
1395703_ 0.654	P	13.39939 A	9.507374 P	11.46878 M	16.81386 A	19.73469 P	15.95647 A	14.46704 A	15.93289 A	18.60787		Transcribed locus
1373350_ 0.652	P	42.7404 P	44.89502 P	40.53615 P	69.90969 P	57.04131 P	71.17689 P	32.93903 P	44.7689 P	43.2724	Psip1	PC4 and SFRS1 interacting protein 1
1389007_ 0.646	M	19.74112 A	20.39906 A	17.73405 P	30.99454 A	26.52852 P	32.64742 A	24.73562 P	21.83741 A	15.13209	RGD1307	Similar to Friedreich ataxia region gene X123 (predicted)
1376084_ 0.645	P	11.72057 P	13.90163 P	11.96186 P	22.00271 P	17.3167 P	19.2781 P	9.522137 A	9.112554 P	22.39788	Espl1_pre	extra spindle poles like 1 (S. cerevisiae) (predicte
1386647_ 0.644	A	16.29533 M	21.98423 A	16.0567 P	32.23388 P	30.31938 P	22.29941 A	21.86999 A	24.88121 A	17.77765	Mtap7_pre	microtubule-associated protein 7 (predicted)
1390126_ 0.639	P	16.16669 P	23.00833 A	16.26274 P	30.01127 P	24.33312 P	32.12049 P	15.50596 A	18.33153 A	14.06883		
1384410_ 0.635	P	6.67688 P	6.029502 P	7.467504 P	10.36879 P	10.50402 P	10.90396 P	6.844386 A	7.720983 M	4.572625		Similar to sorting nexin 12
1389553_ 0.627	P	11.41033 P	14.58559 P	11.39853 P	22.1421 P	16.56908 P	21.28087 P	17.98245 P	9.427206 P	12.37291	RGD:1359	dendritic cell inhibitory receptor 3
1393140_ 0.622	P	19.16512 P	18.57044 P	25.13434 P	30.25585 P	35.72533 P	34.7266 P	22.30524 P	27.24969 P	35.69579		
1380037_ 0.619	A	13.95171 A	11.95194 M	15.17276 P	19.897 A	25.41308 P	21.31351 A	24.61614 A	24.05559 M	19.03213	MGC94040	Hypothetical LOC292764
1379878_ 0.618	P	15.36799 P	11.6994 P	10.39844 P	23.88015 P	19.34261 P	17.35507 P	12.67449 P	15.31673 P	15.76032		Transcribed locus
1370310_ 0.614	P	39.45173 P	52.79944 P	56.41374 P	84.49409 P	71.16423 P	85.27893 P	64.69233 P	48.90293 P	40.04545	Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2
1384309_ 0.614	P	5.183973 M	5.735261 A	6.797849 P	10.47112 P	10.60018 P	7.948514 A	4.876273 A	5.452473 P	8.680175		Transcribed locus
1376784_ 0.61	P	16.68219 P	16.05684 P	22.13426 A	36.56663 A	26.20017 A	27.6394 A	26.69369 P	21.39519 P	22.689	LOC49977	LOC499775
1386486_ 0.608	A	26.06053 P	24.39503 P	32.5661 P	44.18136 P	49.4815 P	42.73749 M	31.94953 P	39.90676 A	22.5611	Rsn	restin (Reed-Steinberg cell-expressed intermediate filament-associated protein)
1382576_ 0.607	P	20.50293 P	24.33367 P	18.71252 P	38.29654 P	30.60166 P	36.09693 P	19.64275 P	31.57876 P	25.9088		CDNA clone IMAGE:7375616
1387408_ 0.605	P	15.49428 P	17.69744 P	20.75498 P	29.2922 P	30.83562 P	28.84386 P	37.21759 P	19.35643 P	16.2496	RGD:62077	seven in absentia 2
1391212_ 0.602	P	17.16262 P	22.42133 P	14.03756 P	32.1649 P	23.10062 P	33.77621 P	21.38269 P	21.8149 P	15.46015	Tceal1	transcription elongation factor A (SII)-like 1
1382902_ 0.6	P	12.69383 P	17.90399 P	14.02534 P	30.58023 P	21.68639 P	22.57047 P	32.91943 P	23.08282 P	13.71616	Herc6	potential ubiquitin ligase
1385916_ 0.599	A	7.920223 P	8.017239 A	9.596525 P	13.65978 P	14.79243 P	14.19691 A	7.409611 A	9.719283 P	15.49892		Transcribed locus
1392139_ 0.596	P	14.59563 P	23.24431 P	18.79842 P	29.61102 P	34.74519 P	29.64774 P	18.50562 P	32.57003 P	40.96218		Transcribed locus
1382810_ 0.595	A	3.912483 A	4.445461 A	4.134687 P	6.941643 P	6.879018 A	7.226104 A	5.360516 A	3.594226 A	7.45944		Similar to Eph receptor A4
1395294_ 0.593	A	13.73278 A	10.15826 A	9.071011 M	22.72719 M	16.94693 P	15.9715 A	13.98234 A	18.1217 P	14.42248		Transcribed locus
1394922_ 0.593	A	9.520434 A	9.265462 A	7.149046 A	17.44643 A	11.11962 P	13.88771 A	14.6159 P	9.753514 A	11.50115		

Appendix 2
Microarray data for all known GH target genes and placenta specific genes containing Stat5b response elements

Probe Set ID	A260_15.CI	A260_17.CI	A260_22.CE	A260_27.CI	A260_28.CE	A260_32.CI	A260_1.CE	A260_4.CE	A260_7.CE	Unigene(Avadis)	Gene Symbol	Gene Title
1368989_a	0.0000	-0.0706	0.0208	0.2003	0.0088	-0.1909	0.0710	-0.5323	-0.1682	Rn.119634	Timp3	tissue inhibitor of metalloproteinase 3
1372926_a	0.0000	0.0251	0.0126	-0.0819	-0.0542	-0.0591	0.0579	-0.0939	0.0097	Rn.119634	Timp3	Tissue inhibitor of metalloproteinase 3
1375138_	0.0000	0.0362	0.0048	-0.1730	-0.1778	-0.1480	0.1010	-0.0110	0.0584	Rn.119634	Timp3	tissue inhibitor of metalloproteinase 3
1386940_a	0.0291	-0.1336	0.1318	0.0686	0.0623	-0.0525	0.0000	-0.3042	-0.0748	Rn.10161	Timp2	tissue inhibitor of metalloproteinase 2
1389836_a_	0.0303	0.0000	0.0229	-0.1264	-0.1859	-0.1134	0.0169	0.0100	-0.0531	Rn.119634	Timp3	Tissue inhibitor of metalloproteinase 3
1377632_a	0.0000	0.0498	-0.0533	0.0313	-0.2678	0.0997	-0.1643	0.0926	-0.1641	Rn.155651	LOC680130	Tissue inhibitor of metalloproteinases-4
1370420_a	-0.0049	0.0556	-0.0486	0.6436	0.5552	-0.1937	0.0000	-0.0729	0.1908	Rn.4620	Srd5a1	steroid 5 alpha-reductase 1
1367533_a	-0.0463	-0.0293	0.1652	0.0762	0.0717	0.0000	-0.0142	-0.0331	0.0749	Rn.25007	Statip1	signal transducer and activator of transcription interacting pro
1368231_a	0.0618	-0.1289	-0.2102	0.1166	0.0000	-0.0385	0.0125	0.0015	-0.0731	Rn.154399	Stat5a	signal transducer and activator of transcription 5A
1368835_a	0.0605	0.0000	0.3097	-0.0658	-0.1084	0.0064	-0.0742	0.0552	-0.0163	Rn.33229	Stat1	signal transducer and activator of transcription 1
1370224_a	0.0760	0.0699	0.0570	0.0000	-0.0651	-0.1194	0.0165	-0.0137	-0.3579	Rn.10247	Stat3	signal transducer and activator of transcription 3
1371781_	-0.0094	0.0611	0.0000	-0.1278	-0.0872	-0.1119	0.1922	0.1775	0.1309	Rn.10247	Stat3	signal transducer and activator of transcription 3
1372757_	0.0294	0.0000	0.1437	0.0959	-0.0913	-0.1499	-0.1229	0.0253	-0.0118	Rn.33229	Stat1	signal transducer and activator of transcription 1
1373670_a	0.0352	-0.0939	-0.1229	0.0000	0.0784	0.0864	-0.1201	0.0154	-0.3431	Rn.24237	Stat2	Signal transducer and activator of transcription 2
1382350_a	0.0240	0.0260	0.0286	-0.0664	0.0000	0.0303	-0.1239	-0.1579	-0.0990	Rn.6880	Stat6_predicted	signal transducer and activator of transcription 6 (predicte
1387354_a	0.0597	-0.1359	0.2629	0.1522	0.0000	0.0380	-0.0669	-0.0539	-0.0129	Rn.33229	Stat1	signal transducer and activator of transcription 1
1387876_a	0.0404	-0.1228	0.1803	0.3339	0.0000	-0.0827	-0.3270	0.0035	-0.1627	Rn.54486	Stat5b	signal transducer and activator of transcription 5B
1389571_	0.1088	0.0000	-0.0038	0.1277	-0.0059	0.0767	-0.3112	0.0025	-0.3673	Rn.24237	Stat2	signal transducer and activator of transcription 2
1384111_	-0.0626	-0.2005	0.0464	0.1128	0.2070	-0.0380	0.0152	0.0000	-0.0441	Rn.137580	Stat4	signal transducer and activator of transcription 4
1369577_	0.0474	0.0106	-0.2227	-0.1039	0.1280	-0.1602	0.1511	-0.0485	0.0000	Rn.205056	Socs2	suppressor of cytokine signaling 2
1369584_a	0.0000	0.0612	-0.0667	0.0031	0.1031	-0.2970	-0.1610	-0.0027	0.1244	Rn.127801	Socs3	suppressor of cytokine signaling 3
1371252_	0.0404	-0.0012	-0.0579	0.0000	-0.0425	0.0712	-0.1323	0.1430	0.1502	Rn.82754	Socs1	suppressor of cytokine signaling 1
1375568_	0.0000	-0.0829	-0.0277	0.0973	0.0103	0.2191	0.0163	-0.0826	-0.0906	Rn.82754	Socs1	Suppressor of cytokine signaling 1
1376666_a	-0.1242	-0.1051	0.1666	0.1795	0.1733	-0.1642	0.0133	-0.1041	0.0000		Socs6_predicted	suppressor of cytokine signaling 6 (predicted)
1377131_	0.0000	-0.2802	-0.0235	0.4723	-0.1386	0.1762	-0.1427	0.1363	0.0464	Rn.205543	Socs7_predicted	Suppressor of cytokine signaling 7 (predicted)
1384888_a	0.0891	0.0548	-0.0446	0.0387	0.0000	0.1938	-0.1751	-0.1412	-0.4526	Rn.205543	Socs7_predicted	suppressor of cytokine signaling 7 (predicted)
1370982_a	0.1434	0.0052	-0.1040	0.1925	-0.1168	-0.0551	0.0000	0.1595	-0.2806	Rn.11238	Pygm	muscle glycogen phosphorylase
1375476_	-0.0836	0.0000	0.1655	-0.2378	-0.0397	0.1298	0.1321	-0.0109	0.1507	Rn.11238	Pygm	muscle glycogen phosphorylase
1383072_a	-0.2132	0.0602	-0.1270	0.0000	-0.0991	0.1968	0.0961	-0.0866	0.0420	Rn.11238	Pygm	muscle glycogen phosphorylase

1371166_	0.1836	-0.1046	0.0337	0.1842	0.0000	-0.0104	-0.4674	0.0183	-0.4040	Rn.44265	Nos3	nitric oxide synthase 3, endothelial cell
1397827_a	0.0000	-0.2038	0.1405	-0.0952	0.0637	-0.0656	-0.1725	0.2836	0.0168	Rn.161711	Nanos3_predicted	nanos homolog 3 (Drosophila) (predicted)
1371237_a	0.1181	-0.4471	0.3966	0.4782	0.6535	0.0000	-0.7602	-0.0101	-0.5047	Rn.54397	Mt1a	metallothionein 1a
1367535_	0.0000	0.0859	-0.1875	-0.0589	-0.0439	0.0360	0.0308	-0.1334	0.0091	Rn.9129	Irf2bp1_predicted	interferon regulatory factor 2 binding protein 1 (pred
1368073_a	-0.0443	-0.0269	0.2112	0.0000	-0.2066	-0.2732	0.3080	0.0351	0.4525	Rn.6396	Irf1	interferon regulatory factor 1
1371560_	-0.0591	-0.1280	0.0000	0.0147	0.1507	0.0615	-0.1511	0.0037	-0.3494	Rn.1499	Irf3	interferon regulatory factor 3
1372097_a	0.0000	-0.3213	0.0518	0.3174	0.0877	-0.2294	-0.3063	0.0791	-0.1127	Rn.3765	Irf8	Interferon regulatory factor 8
1377379_	-0.4591	-0.1399	0.3012	0.2273	-0.1305	-0.5504	0.0000	0.2178	0.4375	Rn.12385	Irf6_predicted	interferon regulatory factor 6 (predicted)
1382503_a	0.0699	0.0000	0.0362	0.2542	0.0798	-0.0655	-0.0233	-0.2330	-0.1921	Rn.107887	Irf2_predicted	interferon regulatory factor 2 (predicted)
1383564_a	0.0000	-0.0408	-0.0780	0.0699	0.1402	0.0566	-0.1457	0.2572	-0.1862	Rn.101159	Irf7	interferon regulatory factor 7
1386518_	0.1117	-0.0769	0.0846	-0.0976	-0.1481	0.0000	0.0026	-0.0126	0.1595	Rn.107887	Irf2_predicted	interferon regulatory factor 2 (predicted)
1386568_a	-0.5544	-0.3516	0.2621	0.0764	-0.1981	-0.7636	0.0678	0.0000	0.2123	Rn.12385	Irf6_predicted	interferon regulatory factor 6 (predicted)
1379563_a	0.0163	-0.0206	0.0484	0.1322	-0.1542	0.0000	0.2515	-0.0772	-0.0183	Rn.203787	Irf5_predicted	interferon regulatory factor 5 (predicted)
1369191_	0.0209	-0.0627	-0.0487	0.0000	-0.0497	-0.1089	0.1039	0.1644	0.1178	Rn.9873	Il6	interleukin 6
1370957_	0.0741	0.0567	0.0000	0.0264	0.0531	-0.2531	-0.2206	-0.1571	-0.3696	Rn.12138	Il6st	interleukin 6 signal transducer
1386987_a	0.0157	-0.1093	0.1228	-0.1501	-0.3641	0.0000	0.3150	-0.3906	0.2093	Rn.1716	Il6ra	interleukin 6 receptor, alpha
1367571_a	-0.0829	0.0019	0.0000	-0.1501	-0.1558	-0.1769	0.0042	0.0022	0.0712	Rn.118681	Igf2	insulin-like growth factor 2
1367636_a	-0.0033	0.1424	0.0000	-0.0946	-0.1216	-0.1679	0.1345	0.1199	0.2027	Rn.270	Igf2r	insulin-like growth factor 2 receptor
1367648_a	0.1049	-0.0179	0.0927	0.1636	0.0282	-0.1150	-0.1390	0.0000	-0.0503	Rn.6813	Igfbp2	insulin-like growth factor binding protein 2
1367652_a	-0.1171	0.1697	0.0000	0.2588	0.3531	-0.1029	-0.3945	-0.5676	0.1083	Rn.26369	Igfbp3	insulin-like growth factor binding protein 3
1368123_a	0.0578	-0.0140	-0.0229	0.0000	0.1424	-0.0032	0.1152	0.0551	-0.1127	Rn.10957	Igf1r	insulin-like growth factor 1 receptor
1368160_a	-1.6178	-1.9061	0.0000	1.7249	0.4240	-1.4182	-0.4862	0.7773	0.3515	Rn.34026	Igfbp1	insulin-like growth factor binding protein 1
1370333_a	0.0000	-0.2672	-0.0019	0.0349	0.0591	0.0924	0.4142	-0.1274	-0.0791	Rn.6282	Igf1	insulin-like growth factor 1
1370960_a	-0.2916	-0.2244	0.0277	0.0404	-0.2289	-0.3758	0.3548	0.0000	0.0090	Rn.1593	Igfbp5	insulin-like growth factor binding protein 5
1371206_a	0.1102	-0.0204	0.0509	0.1483	0.1930	0.0000	-0.1378	-0.0564	-0.1224	Rn.118681	Igf2	insulin-like growth factor 2
1371357_	0.0000	0.1452	-0.0212	-0.1364	-0.1545	-0.2055	0.2113	0.0151	0.3702	Rn.203012	Igfbp7	insulin-like growth factor binding protein 7
1371462_	-0.0058	0.1083	0.0000	0.3008	0.1387	0.0705	-0.0296	-0.0064	-0.2055	Rn.160666	Igfbp4	insulin-like growth factor binding protein 4
1372168_s	-0.0134	-0.1627	0.3434	0.3317	0.2916	0.2007	-0.0034	-0.0336	0.0000	Rn.6431	Igfbp6	insulin-like growth factor binding protein 6
1373882_a	0.0713	0.0283	0.0000	-0.5366	-0.2821	-0.2703	0.1304	-0.0960	0.1271	Rn.10796	Figf	c-fos induced growth factor
1386872_a	0.0947	0.0765	0.0138	-0.0899	-0.0836	-0.1278	-0.0017	0.1497	0.0000	Rn.270	Igf2r	insulin-like growth factor 2 receptor

1386881_a	-0.1139	0.0000	-0.1032	0.2499	0.3364	0.0096	-0.4822	-0.4875	0.0593	Rn.26369	Igfbp3	insulin-like growth factor binding protein 3
1387347_a	0.1648	0.1748	-0.2389	-0.0061	0.2533	-0.0721	0.0000	-0.0344	0.2202	Rn.1593	Igfbp5	insulin-like growth factor binding protein 5
1387348_a	0.0151	0.0191	-0.3287	-0.0176	0.1864	0.0107	0.0000	-0.0011	-0.2672	Rn.1593	Igfbp5	insulin-like growth factor binding protein 5
1387625_a	-0.1026	-0.1540	0.0016	0.2098	0.2827	0.2502	0.0000	-0.2234	-0.2375	Rn.6431	Igfbp6	insulin-like growth factor binding protein 6
1387709_a	0.2245	-0.0452	0.0000	-0.2303	0.0272	0.1153	0.2118	-0.0770	-0.0657	Rn.10796	Figf	c-fos induced growth factor
1387816_	-0.3111	-0.2328	0.5628	0.3294	-0.1053	-0.5583	0.0000	0.3140	0.5831	Rn.7327	Igfals	insulin-like growth factor binding protein, acid labile subunit
1390715_	-0.0103	-0.0877	0.0000	0.0406	0.2791	0.0356	-0.0765	0.1161	-0.1025	Rn.32120	Igfbpl1_predicted	insulin-like growth factor binding protein-like 1 (predicted)
1398322_a	0.0320	0.0574	-0.3151	0.3526	0.1310	0.0000	-0.1339	-0.2462	-0.2210	Rn.118681	Igf2	insulin-like growth factor 2
1377824_a_	-0.0418	0.1881	-0.0492	0.1925	-0.1385	0.0000	0.2335	-0.2140	0.4692	Rn.100446	Igf2bp3	insulin-like growth factor 2, binding protein 3
1382220_a	0.0000	0.0681	-0.0621	-0.0369	0.0558	0.1894	-0.1792	0.0055	-0.2248	Rn.17318	RGD1305614_predicte	similar to IGF-II mRNA-binding protein 2 (predicted)
1382609_a	-0.0425	0.0562	-0.1403	0.1682	0.0921	0.1453	0.0000	-0.0364	-0.0766	Rn.17318	RGD1305614_predicte	Similar to IGF-II mRNA-binding protein 2 (predicted)
1383925_a	0.0297	0.0000	-0.0144	0.0051	0.0037	-0.0528	-0.1656	0.0446	-0.1053	Rn.27362	LOC298643	IGFBP-2-Binding Protein, Ilp45
1385469_a	0.0687	-0.0126	0.1939	0.1318	0.2210	0.0000	-0.0714	-0.0365	-0.0032	Rn.77572	Igf2bp1	insulin-like growth factor 2, binding protein 1
1390860_a	0.0512	0.1118	-0.2526	0.0641	-0.0510	-0.0682	0.0000	-0.0542	0.1897	Rn.100446	Igf2bp3	insulin-like growth factor 2, binding protein 3
1393202_a_	0.0000	-0.1112	0.0045	-0.1182	-0.0640	-0.0576	0.0250	0.1001	0.1092	Rn.100446	Igf2bp3	Insulin-like growth factor 2, binding protein 3
1396152_s_	0.0067	0.0116	-0.1248	0.1623	-0.1808	0.1265	-0.2456	0.0000	-0.0056	Rn.1593	Igfbp5	insulin-like growth factor binding protein 5
1397830_a	0.0893	-0.0948	0.0445	0.3047	0.0000	-0.0264	0.0319	-0.1266	-0.2205	Rn.1593	Igfbp5	insulin-like growth factor binding protein 5
1369289_a	0.0083	0.0981	-0.0200	0.0841	0.0627	-0.0750	0.0000	-0.1045	-0.2220	Rn.44442	Hnf4a	hepatocyte nuclear factor 4, alpha
1382496_a	-0.3026	0.0000	-0.0046	0.2372	0.1600	0.1004	0.0078	-0.1198	-0.0552	Rn.44442	Hnf4a	hepatocyte nuclear factor 4, alpha
1379329_a	-0.0055	0.0086	0.0725	-0.1564	-0.0981	0.0000	0.0934	-0.1779	0.1965		LOC683319	similar to Pterin-4-alpha-carbinolamine dehydratase 2 (PHS 2)
1379330_s_	-0.0226	0.0079	0.1270	-0.1066	-0.0301	0.0000	0.2138	-0.1438	0.2795		LOC683319	similar to Pterin-4-alpha-carbinolamine dehydratase 2 (PHS 2)
1390918_a	0.0000	0.2974	0.4137	-0.4423	-0.5067	-0.1612	0.7262	-0.0265	0.8657	Rn.19589	Grtp1	GH regulated TBC protein 1
1368395_a	-0.4079	-0.3981	0.1194	0.1781	-0.1814	-0.5675	0.0663	0.0000	0.3003	Rn.9717	Gpc3	glypican 3
1368786_a_	0.0000	-0.0058	-0.2014	0.2064	0.1494	0.0734	-0.0018	0.0549	-0.1518	Rn.88417	Gpcr12	G-protein coupled receptor 12
1369055_a	-0.2896	-0.2481	0.1961	0.2166	0.0000	-0.2342	0.2654	-0.0694	0.3818	Rn.64507	Edg7	putative G protein-coupled receptor snGPCR32
1370448_a	0.0334	-0.0495	-0.0528	0.1293	0.0733	0.0000	-0.0866	0.1242	-0.1985	Rn.88131	Gpc2	glypican 2 (cerebroglycan)
1387039_a	0.0005	-0.0681	0.0000	-0.4101	-0.2968	0.0432	0.1469	0.1985	-0.2473	Rn.7044	Gpc1	glypican 1
1378554_	-0.0376	-0.0118	-0.1206	0.0000	0.1909	0.2285	0.1951	-0.1453	0.1534	Rn.141072		
1379821_	-0.2353	0.0000	0.2050	0.0427	0.0948	-0.5618	-0.1005	0.0814	-0.3710		RGD1560166_predicte	similar to Probable G-protein coupled receptor 62 (hGPCR8) (predicted)
1392888_a	-0.0311	-0.1111	-0.1303	0.0234	0.0000	-0.3302	0.1903	0.0332	0.1117	Rn.19945	Gpc4	glypican 4

1368947_a	0.0528	0.0201	-0.0627	-0.1091	-0.0473	0.0000	0.0244	-0.0339	0.0513	Rn.10250	Gadd45a	growth arrest and DNA-damage-inducible 45 alpha
1371896_	-0.0628	-0.0971	0.0442	-0.0260	-0.0062	0.1109	0.0000	0.0551	0.0099	Rn.161715	Gadd45gip1	growth arrest and DNA-damage-inducible, gamma interacting protein
1372016_	0.0985	0.0762	0.1374	0.0000	-0.0707	-0.1336	-0.0189	0.1527	-0.0857	Rn.35886	Gadd45b	growth arrest and DNA-damage-inducible 45 beta
1388792_a	-0.0772	-0.0530	0.0928	-0.1088	0.0058	0.0000	0.0221	-0.1569	0.1285	Rn.16950	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma
1380605_a	0.1088	-0.0872	0.0703	-0.0193	-0.0741	0.0639	0.2103	0.0000	-0.0874	Rn.173545		
1368489_a	0.0000	-0.3051	0.0543	0.3376	0.0992	-0.0182	-0.3795	0.0877	-0.2350	Rn.11306	Fosl1	fos-like antigen 1
1373882_a	0.0713	0.0283	0.0000	-0.5366	-0.2821	-0.2703	0.1304	-0.0960	0.1271	Rn.10796	Figf	c-fos induced growth factor
1375043_a	-0.1094	0.0191	0.0000	0.0127	-0.0649	-0.2524	0.2200	-0.0541	0.2721	Rn.103750	Fos	FBJ murine osteosarcoma viral oncogene homolog
1387530_a	0.2838	-0.0161	0.4089	0.4766	0.2800	-0.1452	-0.0326	0.0000	-0.0039	Rn.163577	Fosb /// Fosl2	fos-like antigen 2 /// FBJ osteosarcoma oncogene B
1387709_a	0.2245	-0.0452	0.0000	-0.2303	0.0272	0.1153	0.2118	-0.0770	-0.0657	Rn.10796	Figf	c-fos induced growth factor
1383860_a	0.1543	-0.2991	0.0000	0.1811	0.0013	-0.3393	-0.3860	0.0165	-0.5003	Rn.163577	Fosl2	Fos-like antigen 2
1370234_a	0.0000	-0.0269	0.0022	0.0687	0.0434	-0.0194	-0.0556	0.0034	-0.0638	Rn.1604	Fn1	fibronectin 1
1373206_a	0.0000	0.0747	-0.0021	0.0348	0.0234	-0.0231	-0.0234	-0.0364	0.0413	Rn.757	Fndc3b_predicted	fibronectin type III domain containing 3B (predicted)
1373416_	-0.0526	0.1412	0.0000	-0.0165	0.1291	-0.1346	0.0642	-0.1591	0.1027	Rn.757	Fndc3b_predicted	fibronectin type III domain containing 3B (predicted)
1374726_a	0.1154	0.1990	-0.1419	-0.0090	-0.2650	0.0000	0.3778	-0.1981	0.2913	Rn.20633	Fndc1	fibronectin type III domain containing 1
1376532_a	-0.0471	0.0385	-0.0686	0.0167	0.0715	0.0736	-0.1531	-0.1321	0.0000	Rn.757	Fndc3b_predicted	fibronectin type III domain containing 3B (predicted)
1376823_a	0.0000	-0.0228	-0.0463	0.1263	0.1767	0.1088	0.0488	-0.1306	-0.1354	Rn.100627	Fndc3a_predicted	Fibronectin type III domain containing 3a (predicted)
1378057_	0.0078	0.0000	-0.1819	0.1858	0.1687	-0.0898	-0.0558	-0.0942	0.0113	Rn.95324	Flrt3_predicted	fibronectin leucine rich transmembrane protein 3 (predicted)
1379921_	-0.2361	0.0325	-0.1188	0.0000	0.0363	-0.0240	-0.1002	0.1108	0.0244	Rn.41372	Fsd2_predicted	fibronectin type III and SPRY domain containing 2 (predicted)
1382638_a	-0.1865	0.0963	0.0000	0.2456	-0.2232	-0.2050	0.1697	-0.1049	0.0226	Rn.95324	Flrt3_predicted	Fibronectin leucine rich transmembrane protein 3 (predicted)
1383396_a	-0.1328	-0.0575	0.2087	-0.0264	0.1294	0.0469	0.0000	-0.0145	0.2625	Rn.100627	Fndc3a_predicted	fibronectin type III domain containing 3a (predicted)
1392863_a	-0.0706	-0.0254	-0.1935	0.0763	0.1959	0.0000	0.0314	-0.2353	0.1642	Rn.95324	Flrt3_predicted	fibronectin leucine rich transmembrane protein 3 (predicted)
1367660_a	0.0057	0.1426	0.0000	-0.1344	-0.0053	-0.0208	-0.0017	0.1720	0.1148	Rn.32566	Fabp3	fatty acid binding protein 3
1368271_a	0.1509	-0.1205	0.0863	-0.0751	-0.0109	0.2974	0.1421	-0.2433	0.0000	Rn.4258	Fabp4	fatty acid binding protein 4, adipocyte
1368630_a	0.0000	0.1845	-0.0509	-0.1134	0.0097	0.0690	0.0622	-0.1267	-0.0381	Rn.10078	Fabp9	fatty acid binding protein 9, testis
1368697_a	0.0238	-0.0681	0.0000	-0.0729	-0.1237	-0.1452	0.0427	0.0232	0.0213	Rn.10008	Fabp6	fatty acid binding protein 6, ileal (gastrotropin)
1369111_	0.0488	0.0000	-0.0292	0.1265	-0.1104	0.0498	-0.0417	-0.0511	0.0221	Rn.36412	Fabp1	fatty acid binding protein 1, liver
1369195_	-0.1937	-0.2941	0.2696	0.1901	0.1936	-0.0911	0.0000	0.0317	-0.0674	Rn.91358	Fabp2	fatty acid binding protein 2, intestinal
1370024_a	0.0201	-0.0743	0.0147	-0.0457	0.0463	-0.1145	0.0989	0.0000	-0.0530	Rn.10014	Fabp7	fatty acid binding protein 7, brain
1370281_	0.1084	0.0000	-0.0476	0.0847	0.1436	0.0204	-0.0410	-0.0170	-0.0614	Rn.98269	Fabp5	fatty acid binding protein 5, epidermal

1375568_	0.0000	-0.0829	-0.0277	0.0973	0.0103	0.2191	0.0163	-0.0826	-0.0906	Rn.82754	Socs1	Suppressor of cytokine signaling 1
1376666_a	-0.1242	-0.1051	0.1666	0.1795	0.1733	-0.1642	0.0133	-0.1041	0.0000		Socs6_predicted	suppressor of cytokine signaling 6 (predicted)
1377131_	0.0000	-0.2802	-0.0235	0.4723	-0.1386	0.1762	-0.1427	0.1363	0.0464	Rn.205543	Socs7_predicted	Suppressor of cytokine signaling 7 (predicted)
1384888_a	0.0891	0.0548	-0.0446	0.0387	0.0000	0.1938	-0.1751	-0.1412	-0.4526	Rn.205543	Socs7_predicted	suppressor of cytokine signaling 7 (predicted)
1370982_a	0.1434	0.0052	-0.1040	0.1925	-0.1168	-0.0551	0.0000	0.1595	-0.2806	Rn.11238	Pygm	muscle glycogen phosphorylase
1375476_	-0.0836	0.0000	0.1655	-0.2378	-0.0397	0.1298	0.1321	-0.0109	0.1507	Rn.11238	Pygm	muscle glycogen phosphorylase
1383072_a	-0.2132	0.0602	-0.1270	0.0000	-0.0991	0.1968	0.0961	-0.0866	0.0420	Rn.11238	Pygm	muscle glycogen phosphorylase
1371166_	0.1836	-0.1046	0.0337	0.1842	0.0000	-0.0104	-0.4674	0.0183	-0.4040	Rn.44265	Nos3	nitric oxide synthase 3, endothelial cell
1397827_a	0.0000	-0.2038	0.1405	-0.0952	0.0637	-0.0656	-0.1725	0.2836	0.0168	Rn.161711	Nanos3_predicted	nanos homolog 3 (Drosophila) (predicted)
1371237_a	0.1181	-0.4471	0.3966	0.4782	0.6535	0.0000	-0.7602	-0.0101	-0.5047	Rn.54397	Mt1a	metallothionein 1a
1367535_	0.0000	0.0859	-0.1875	-0.0589	-0.0439	0.0360	0.0308	-0.1334	0.0091	Rn.9129	Irf2bp1_predicte	interferon regulatory factor 2 binding protein 1 (predic
1368073_a	-0.0443	-0.0269	0.2112	0.0000	-0.2066	-0.2732	0.3080	0.0351	0.4525	Rn.6396	Irf1	interferon regulatory factor 1
1371560_	-0.0591	-0.1280	0.0000	0.0147	0.1507	0.0615	-0.1511	0.0037	-0.3494	Rn.1499	Irf3	interferon regulatory factor 3
1372097_a	0.0000	-0.3213	0.0518	0.3174	0.0877	-0.2294	-0.3063	0.0791	-0.1127	Rn.3765	Irf8	Interferon regulatory factor 8
1377379_	-0.4591	-0.1399	0.3012	0.2273	-0.1305	-0.5504	0.0000	0.2178	0.4375	Rn.12385	Irf6_predicted	interferon regulatory factor 6 (predicted)
1382503_a	0.0699	0.0000	0.0362	0.2542	0.0798	-0.0655	-0.0233	-0.2330	-0.1921	Rn.107887	Irf2_predicted	interferon regulatory factor 2 (predicted)
1383564_a	0.0000	-0.0408	-0.0780	0.0699	0.1402	0.0566	-0.1457	0.2572	-0.1862	Rn.101159	Irf7	interferon regulatory factor 7
1386518_	0.1117	-0.0769	0.0846	-0.0976	-0.1481	0.0000	0.0026	-0.0126	0.1595	Rn.107887	Irf2_predicted	interferon regulatory factor 2 (predicted)
1386568_a	-0.5544	-0.3516	0.2621	0.0764	-0.1981	-0.7636	0.0678	0.0000	0.2123	Rn.12385	Irf6_predicted	interferon regulatory factor 6 (predicted)
1379563_a	0.0163	-0.0206	0.0484	0.1322	-0.1542	0.0000	0.2515	-0.0772	-0.0183	Rn.203787	Irf5_predicted	interferon regulatory factor 5 (predicted)
1369191_	0.0209	-0.0627	-0.0487	0.0000	-0.0497	-0.1089	0.1039	0.1644	0.1178	Rn.9873	Il6	interleukin 6
1370957_	0.0741	0.0567	0.0000	0.0264	0.0531	-0.2531	-0.2206	-0.1571	-0.3696	Rn.12138	Il6st	interleukin 6 signal transducer
1386987_a	0.0157	-0.1093	0.1228	-0.1501	-0.3641	0.0000	0.3150	-0.3906	0.2093	Rn.1716	Il6ra	interleukin 6 receptor, alpha
1367571_a	-0.0829	0.0019	0.0000	-0.1501	-0.1558	-0.1769	0.0042	0.0022	0.0712	Rn.118681	Igf2	insulin-like growth factor 2
1367636_a	-0.0033	0.1424	0.0000	-0.0946	-0.1216	-0.1679	0.1345	0.1199	0.2027	Rn.270	Igf2r	insulin-like growth factor 2 receptor
1367648_a	0.1049	-0.0179	0.0927	0.1636	0.0282	-0.1150	-0.1390	0.0000	-0.0503	Rn.6813	Igfbp2	insulin-like growth factor binding protein 2
1367652_a	-0.1171	0.1697	0.0000	0.2588	0.3531	-0.1029	-0.3945	-0.5676	0.1083	Rn.26369	Igfbp3	insulin-like growth factor binding protein 3
1368123_a	0.0578	-0.0140	-0.0229	0.0000	0.1424	-0.0032	0.1152	0.0551	-0.1127	Rn.10957	Igf1r	insulin-like growth factor 1 receptor
1368160_a	-1.6178	-1.9061	0.0000	1.7249	0.4240	-1.4182	-0.4862	0.7773	0.3515	Rn.34026	Igfbp1	insulin-like growth factor binding protein 1
1370333_a_	0.0000	-0.2672	-0.0019	0.0349	0.0591	0.0924	0.4142	-0.1274	-0.0791	Rn.6282	Igf1	insulin-like growth factor 1

1368786_a_	0.0000	-0.0058	-0.2014	0.2064	0.1494	0.0734	-0.0018	0.0549	-0.1518	Rn.88417	Gpcr12	G-protein coupled receptor 12
1369055_a	-0.2896	-0.2481	0.1961	0.2166	0.0000	-0.2342	0.2654	-0.0694	0.3818	Rn.64507	Edg7	putative G protein-coupled receptor snGPCR32
1370448_a	0.0334	-0.0495	-0.0528	0.1293	0.0733	0.0000	-0.0866	0.1242	-0.1985	Rn.88131	Gpc2	glypican 2 (cerebroglycan)
1387039_a	0.0005	-0.0681	0.0000	-0.4101	-0.2968	0.0432	0.1469	0.1985	-0.2473	Rn.7044	Gpc1	glypican 1
1378554_at	-0.0376	-0.0118	-0.1206	0.0000	0.1909	0.2285	0.1951	-0.1453	0.1534	Rn.141072		Transcribed locus, strongly similar to XP_980173.1
1379821_at	-0.2353	0.0000	0.2050	0.0427	0.0948	-0.5618	-0.1005	0.0814	-0.3710		RGD1560166_predicte	similar to Probable G-protein coupled receptor 62 (hGPCR8) (predicted
1392888_at	-0.0311	-0.1111	-0.1303	0.0234	0.0000	-0.3302	0.1903	0.0332	0.1117	Rn.19945	Gpc4	glypican 4
1368947_at	0.0528	0.0201	-0.0627	-0.1091	-0.0473	0.0000	0.0244	-0.0339	0.0513	Rn.10250	Gadd45a	growth arrest and DNA-damage-inducible 45 alpha
1371896_at	-0.0628	-0.0971	0.0442	-0.0260	-0.0062	0.1109	0.0000	0.0551	0.0099	Rn.161715	Gadd45gip1	growth arrest and DNA-damage-inducible, gamma interacting protein
1372016_at	0.0985	0.0762	0.1374	0.0000	-0.0707	-0.1336	-0.0189	0.1527	-0.0857	Rn.35886	Gadd45b	growth arrest and DNA-damage-inducible 45 beta
1388792_at	-0.0772	-0.0530	0.0928	-0.1088	0.0058	0.0000	0.0221	-0.1569	0.1285	Rn.16950	Gadd45g	growth arrest and DNA-damage-inducible 45 gamma
1380605_at	0.1088	-0.0872	0.0703	-0.0193	-0.0741	0.0639	0.2103	0.0000	-0.0874	Rn.173545		Transcribed locus, strongly similar to XP_213842.2
1368489_at	0.0000	-0.3051	0.0543	0.3376	0.0992	-0.0182	-0.3795	0.0877	-0.2350	Rn.11306	Fosl1	fos-like antigen 1
1373882_at	0.0713	0.0283	0.0000	-0.5366	-0.2821	-0.2703	0.1304	-0.0960	0.1271	Rn.10796	Figf	c-fos induced growth factor
1375043_at	-0.1094	0.0191	0.0000	0.0127	-0.0649	-0.2524	0.2200	-0.0541	0.2721	Rn.103750	Fos	FBJ murine osteosarcoma viral oncogene homolog
1387530_a_at	0.2838	-0.0161	0.4089	0.4766	0.2800	-0.1452	-0.0326	0.0000	-0.0039	Rn.163577	Fosb /// Fosl2	fos-like antigen 2 /// FBJ osteosarcoma oncogene B
1387709_at	0.2245	-0.0452	0.0000	-0.2303	0.0272	0.1153	0.2118	-0.0770	-0.0657	Rn.10796	Figf	c-fos induced growth factor
1383860_at	0.1543	-0.2991	0.0000	0.1811	0.0013	-0.3393	-0.3860	0.0165	-0.5003	Rn.163577	Fosl2	Fos-like antigen 2
1370234_at	0.0000	-0.0269	0.0022	0.0687	0.0434	-0.0194	-0.0556	0.0034	-0.0638	Rn.1604	Fn1	fibronectin 1
1373206_at	0.0000	0.0747	-0.0021	0.0348	0.0234	-0.0231	-0.0234	-0.0364	0.0413	Rn.757	Fndc3b_predicted	fibronectin type III domain containing 3B (predicted)
1373416_at	-0.0526	0.1412	0.0000	-0.0165	0.1291	-0.1346	0.0642	-0.1591	0.1027	Rn.757	Fndc3b_predicted	fibronectin type III domain containing 3B (predicted)
1374726_at	0.1154	0.1990	-0.1419	-0.0090	-0.2650	0.0000	0.3778	-0.1981	0.2913	Rn.20633	Fndc1	fibronectin type III domain containing 1
1376532_at	-0.0471	0.0385	-0.0686	0.0167	0.0715	0.0736	-0.1531	-0.1321	0.0000	Rn.757	Fndc3b_predicted	fibronectin type III domain containing 3B (predicted)
1376823_at	0.0000	-0.0228	-0.0463	0.1263	0.1767	0.1088	0.0488	-0.1306	-0.1354	Rn.100627	Fndc3a_predicted	Fibronectin type III domain containing 3a (predicted)
1378057_at	0.0078	0.0000	-0.1819	0.1858	0.1687	-0.0898	-0.0558	-0.0942	0.0113	Rn.95324	Flrt3_predicted	fibronectin leucine rich transmembrane protein 3 (predicted)
1379921_at	-0.2361	0.0325	-0.1188	0.0000	0.0363	-0.0240	-0.1002	0.1108	0.0244	Rn.41372	Fsd2_predicted	fibronectin type III and SPRY domain containing 2 (predicted)
1382638_at	-0.1865	0.0963	0.0000	0.2456	-0.2232	-0.2050	0.1697	-0.1049	0.0226	Rn.95324	Flrt3_predicted	Fibronectin leucine rich transmembrane protein 3 (predicted)
1383396_at	-0.1328	-0.0575	0.2087	-0.0264	0.1294	0.0469	0.0000	-0.0145	0.2625	Rn.100627	Fndc3a_predicted	fibronectin type III domain containing 3a (predicted)
1392863_at	-0.0706	-0.0254	-0.1935	0.0763	0.1959	0.0000	0.0314	-0.2353	0.1642	Rn.95324	Flrt3_predicted	fibronectin leucine rich transmembrane protein 3 (predicted)
1367660_at	0.0057	0.1426	0.0000	-0.1344	-0.0053	-0.0208	-0.0017	0.1720	0.1148	Rn.32566	Fabp3	fatty acid binding protein 3

1368271_a_at	0.1509	-0.1205	0.0863	-0.0751	-0.0109	0.2974	0.1421	-0.2433	0.0000	Rn.4258	Fabp4	fatty acid binding protein 4, adipocyte
1368630_at	0.0000	0.1845	-0.0509	-0.1134	0.0097	0.0690	0.0622	-0.1267	-0.0381	Rn.10078	Fabp9	fatty acid binding protein 9, testis
1368697_at	0.0238	-0.0681	0.0000	-0.0729	-0.1237	-0.1452	0.0427	0.0232	0.0213	Rn.10008	Fabp6	fatty acid binding protein 6, ileal (gastrotropin)
1369111_at	0.0488	0.0000	-0.0292	0.1265	-0.1104	0.0498	-0.0417	-0.0511	0.0221	Rn.36412	Fabp1	fatty acid binding protein 1, liver
1369195_at	-0.1937	-0.2941	0.2696	0.1901	0.1936	-0.0911	0.0000	0.0317	-0.0674	Rn.91358	Fabp2	fatty acid binding protein 2, intestinal
1370024_at	0.0201	-0.0743	0.0147	-0.0457	0.0463	-0.1145	0.0989	0.0000	-0.0530	Rn.10014	Fabp7	fatty acid binding protein 7, brain
1370281_at	0.1084	0.0000	-0.0476	0.0847	0.1436	0.0204	-0.0410	-0.0170	-0.0614	Rn.98269	Fabp5	fatty acid binding protein 5, epidermal
1370699_a_at	-0.3145	-0.2400	0.2001	0.1170	-0.0110	-0.2736	0.0714	0.0000	0.2603	Rn.37227	Egfr /// Pepd	epidermal growth factor receptor /// peptidase D
1370830_at	0.2342	-0.0383	-0.0354	0.0776	0.0343	-0.2331	0.2681	0.0000	-0.2253	Rn.37227	Egfr	epidermal growth factor receptor
1385413_at	0.2990	-0.0448	0.0990	0.0000	-0.0637	-0.0637	0.1302	-0.2061	0.1417	Rn.37227	Egfr	epidermal growth factor receptor
1367988_at	-0.5116	-0.5098	0.4797	0.7868	0.0000	-0.2896	-0.3307	0.0662	0.1668	Rn.2184	Cyp2c23	cytochrome P450, family 2, subfamily c, polypeptide 23
1368155_at	0.0861	-0.0412	-0.0578	0.0118	0.0116	0.0000	-0.1547	0.1466	-0.0528	Rn.2586	Cyp2c12	cytochrome P450, family 2, subfamily c, polypeptide 12
1368468_at	0.0799	0.0000	0.1524	0.0009	-0.2575	0.1280	-0.0942	-0.2308	-0.1754	Rn.1401	Cyp11a1	cytochrome P450, family 11, subfamily a, polypeptide 1
1368636_at	0.0000	-0.0255	0.0230	-0.1717	0.0838	-0.0177	-0.0144	0.0225	0.0633	Rn.10847	Cyp27b1	cytochrome P450, family 27, subfamily b, polypeptide 1
1368738_at	0.0823	-0.1111	-0.0956	0.0000	0.0364	-0.1043	0.0272	0.1620	-0.1663	Rn.198235	Cyp11b1	cytochrome P450, subfamily 11B, polypeptide 1
1368739_s_at	0.0368	0.1239	0.0315	0.0000	-0.0644	-0.1354	0.0167	-0.1164	-0.1185	Rn.198236	Cyp11b1 /// Cyp11b2 /// Cyp	cytochrome P450, family 11, subfamily B, polypeptide 2 , polypeptide 3 , polypepti
1368990_at	-0.0028	0.0635	0.0212	0.0000	0.2733	0.3023	-0.0437	-0.1989	-0.3639	Rn.10125	Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1
1369136_at	0.0208	-0.1603	0.0233	0.0424	0.1076	0.0000	-0.2185	-0.3179	-0.3485	Rn.2063	Cyp2a3a	cytochrome P450, family 2, subfamily A, polypeptide 3a
1369264_at	-0.0421	0.1396	-0.0611	-0.1468	0.0384	0.0000	0.0049	0.0506	-0.0400	Rn.161726	Cyp21a1	cytochrome P450, subfamily 21A, polypeptide 1
1369275_s_at	-0.0273	0.0617	-0.1428	-0.0027	-0.0662	0.1313	0.0938	0.0131	0.0000	Rn.9867	Cyp2a1 /// Cyp2a2	cytochrome P450 IIA1
1369424_at	0.0000	0.0047	0.1426	0.0386	-0.0002	0.2579	-0.0002	-0.0002	-0.0002	Rn.9867	Cyp2a2	cytochrome P450, subfamily 2A, polypeptide 1
1369444_at	-0.1153	-0.1645	0.0252	0.0338	0.0484	0.0000	-0.0589	-0.0665	0.0386	Rn.21402	Cyp19a1	cytochrome P450, family 19, subfamily a, polypeptide 1
1370241_at	0.1879	0.0594	-0.2864	-0.3244	0.1088	-0.0949	0.1207	0.0000	-0.2239	Rn.1247	Cyp2c7	cytochrome P450, family 2, subfamily c, polypeptide 7
1370269_at	-0.0676	-0.2051	-0.0279	0.4732	0.5083	0.0175	-0.1119	0.3176	0.0000	Rn.10352	Cyp1a1	cytochrome P450, family 1, subfamily a, polypeptide 1
1370475_at	0.0491	-0.0438	0.2407	0.2919	-0.1354	0.1581	-0.0893	0.0000	-0.0042	Rn.4845	Cyp2b3	cytochrome P450IIB3
1370495_s_at	-0.0271	-0.0550	-0.1799	0.0914	0.2743	-0.2155	0.0011	0.0000	0.2019	Rn.82715	Cyp2c13	cytochrome P450 2c13
1370497_at	-0.0485	0.0681	0.0814	-0.0550	0.0163	0.0539	-0.0558	0.0000	-0.1702	Rn.198236	Cyp11b3	cytochrome P450, subfamily 11B, polypeptide 3
1371076_at	0.0554	-0.0860	0.0000	0.0780	0.2645	-0.1485	-0.0023	0.1779	-0.0055	Rn.91353	Cyp2b15 /// Cyp2b2	cytochrome P450, family 2, subfamily b, polypeptide 15, polypeptide 2
1376667_at	0.0000	-0.4174	0.0067	-0.0528	0.0649	-0.1118	0.3276	0.1508	-0.1670	Rn.19898	Cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1
1387123_at	0.2472	-0.1063	0.2602	0.1158	-0.1609	0.0000	-0.0147	0.0607	-0.4130	Rn.10172	Cyp17a1	cytochrome P450, family 17, subfamily a, polypeptide 1

1387243_at	-0.1882	0.0046	-0.1980	0.1699	0.0000	-0.0391	0.0011	-0.0408	0.0216	Rn.5563	Cyp1a2	cytochrome P450, family 1, subfamily a, polypeptide 2
1387305_s_at	-0.0395	0.0000	-0.0793	0.2549	0.0298	0.1126	0.1638	-0.1583	-0.1372	Rn.198235	Cyp11b1 /// Cyp11b2	cytochrome P450, family 11, subfamily B, polypeptide 2, polypeptide
1387328_at	0.0270	0.0347	-0.2283	-0.0833	0.0625	0.0000	-0.1982	0.0424	-0.2745	Rn.10870	Cyp2c	cytochrome P450, subfamily IIC (mephenytoin 4-hydroxylase)
1387511_at	0.0000	-0.0255	-0.0952	-0.1118	0.1915	0.0612	0.2545	-0.4288	0.0560	Rn.32107	Cyp2a1	cytochrome P450 IIA1 (hepatic steroid hydroxylase IIA1) gene
1387583_at	-0.0736	0.0989	-0.0966	-0.0475	0.0725	-0.1860	0.1559	0.3419	0.0000	Rn.81072	Cyp26a1	cytochrome P450, family 26, subfamily A, polypeptide 1
1387722_at	0.0947	0.0616	-0.1781	0.0000	-0.1499	0.1137	-0.0993	0.0535	-0.0903	Rn.144570	Cyp2b15	cytochrome P450, family 2, subfamily b, polypeptide 15
1387914_at	-0.0679	-0.2633	0.4012	0.1428	0.0894	-0.2719	0.0000	-0.0281	0.2327	Rn.94956	Cyp27a1	cytochrome P450, family 27, subfamily a, polypeptide 1
1387949_at	-1.2063	-1.4705	0.4483	0.4797	0.0000	-1.0213	-0.3387	0.0356	0.2922	Rn.88025	Cyp2c22	cytochrome P450, family 2, subfamily c, polypeptide 22
1387993_at	0.0680	0.0000	0.1599	-0.0974	0.0090	0.0642	-0.1365	-0.0921	-0.1771	Rn.38261	Cyp2b21	cytochrome P450, family 2, subfamily b, polypeptide 21
1378551_at	-0.1581	-0.1159	-0.0059	0.0000	0.0418	-0.0792	0.1267	0.0859	0.1140	Rn.101629	Cyp20a1	cytochrome P450, family 20, subfamily A, polypeptide 1
1384392_at	-0.2805	-0.3522	0.1840	-0.0249	0.3279	0.0000	0.5382	0.1078	-0.0535	Rn.19898	Cyp26b1	cytochrome P450, family 26, subfamily b, polypeptide 1
1369354_at	0.0000	-0.2202	0.2189	0.5552	-0.0427	-0.0963	0.2080	0.4266	-0.0988	Rn.83632	Csf1	colony stimulating factor 1 (macrophage)
1369529_at	0.1554	-0.4372	0.1481	-0.0837	0.0738	0.1726	-0.1034	0.0000	-0.0961	Rn.53973	Csf3	colony stimulating factor 3 (granulocyte)
1369828_at	0.1611	-0.0131	-0.2885	0.0077	0.1137	0.0994	-0.1221	0.0000	-0.0025	Rn.42930	Csf2rb1	colony stimulating factor 2 receptor, beta 1
1371227_at	0.0886	0.0000	-0.1690	0.0008	0.0185	-0.1077	-0.0418	0.0138	-0.1159	Rn.44285	Csf2	colony stimulating factor 2 (granulocyte-macrophage)
1371228_at	0.2749	-0.0297	0.0234	-0.2109	-0.1051	0.0000	-0.0370	0.0488	0.0566	Rn.44285	Csf2	colony stimulating factor 2 (granulocyte-macrophage)
1373020_at	0.0020	0.0082	-0.1041	0.0305	0.0506	-0.1043	-0.1265	0.0000	-0.1154	Rn.144694	LOC679907 /// LOC68	similar to mitochondria-associated granulocyte macrophage CSF signaling mol
1388784_at	0.0000	0.0301	-0.0391	-0.4358	-0.3435	-0.0211	0.2865	0.0364	0.0841	Rn.72599	Csf1r	colony stimulating factor 1 receptor
1379631_at	-0.0902	0.1234	0.0000	-0.0110	0.2408	-0.4566	0.0560	-0.0391	0.1589	Rn.83632	Csf1	colony stimulating factor 1 (macrophage)
1380582_at	0.1466	0.0777	0.0000	0.1118	0.1163	-0.0036	-0.0278	-0.0503	-0.2263	Rn.83632	Csf1	colony stimulating factor 1 (macrophage)
1380583_s_at	0.0726	0.0832	-0.1144	-0.0222	0.1073	0.0108	-0.0477	0.0000	-0.2442	Rn.83632	Csf1	colony stimulating factor 1 (macrophage)
1382153_at	-0.1235	0.2176	-0.0129	-0.0370	0.0000	-0.1967	0.2079	0.0046	0.1807	Rn.24032	Clecsf6	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily
1382692_at	0.1667	0.1048	0.1820	-0.0920	-0.0541	-0.0740	0.2159	0.0000	-0.0883	Rn.15743	RGD1565140_predicte	similar to Clecsf12 protein (predicted)
1386009_at	-0.0142	0.0990	-0.1242	0.0187	0.0043	-0.1953	0.1304	-0.0937	0.0000	Rn.134664	Csf3r_predicted	colony stimulating factor 3 receptor (granulocyte) (predicted)
1392528_at	0.1054	0.1318	0.0000	0.0063	-0.0550	-0.0002	0.2189	-0.1239	-0.2701	Rn.216620	Csf2ra	Granulocyte-macrophage colony stimulating receptor alpha
1397360_at	0.0000	0.0737	0.0452	-0.0533	0.0807	-0.0442	-0.1258	0.0442	-0.0542	Rn.82624	Clecsf1_predicted	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamil
1372481_at	0.0000	-0.2220	0.4455	0.2511	0.4987	0.1469	-0.4029	-0.2281	-0.2576	Rn.2721	Cd34_predicted	CD34 antigen (predicted)
1370131_at	-0.0752	0.1155	-0.0080	0.0000	0.0261	-0.2153	0.2791	-0.5422	0.5955	Rn.22518	Cav1	caveolin, caveolae protein 1
1372111_at	0.0000	0.0323	-0.0990	-0.0341	0.0600	-0.1070	0.1606	-0.0655	0.2309	Rn.22518	Cav1	caveolin, caveolae protein 1
1393281_at	0.0000	0.0895	-0.1152	-0.1287	0.0623	-0.1566	0.2416	-0.2845	0.4642	Rn.22518	Cav1	caveolin, caveolae protein 1

1388544_at	0.1119	0.0000	-0.0114	-0.0457	0.0551	0.2872	0.1223	-0.0139	-0.1097	Rn.204528	Bpgm	2,3-bisphosphoglycerate mutase
1383551_at	-0.0305	0.0288	-0.0288	-0.0496	0.0522	0.1683	0.2099	-0.1876	0.0000	Rn.204528	Bpgm	2,3-bisphosphoglycerate mutase
1377166_at	-0.0006	0.0000	0.0322	-0.1048	0.0480	0.0179	-0.0105	-0.0723	0.0353	Rn.6408	Als2	Amyotrophic lateral sclerosis 2 (juvenile) homolog (human)
1386313_x_at	0.3000	-0.0265	0.3642	-0.0266	0.0862	0.0000	-0.0143	-0.0241	0.2345	Rn.98599	Als2cr4_predicted	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 4 (predict
1398562_at	0.0040	-0.2301	0.1461	0.0000	-0.1889	-0.0443	0.2154	-0.0211	0.1873	Rn.98599	Als2cr4_predicted	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 4 (predict
1381455_at	-0.0247	-0.0805	0.1136	-0.0220	-0.0689	0.0000	0.2494	0.2358	0.0740	Rn.98599	Als2cr4_predicted	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 4 (predict
1383155_at	0.0456	-0.2303	0.0213	-0.1206	0.0069	-0.0382	0.0887	0.0000	-0.0587	Rn.8070	Als2cr13_predicted	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 13 (predi
1384279_at	0.1391	0.0000	-0.0296	0.3322	-0.0581	-0.1160	0.2948	-0.0147	0.3831	Rn.8070	Als2cr13_predicted	amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 13 (predi
1367555_at	0.0000	0.0040	-0.4016	0.2933	0.0943	-0.3539	-0.0303	0.2045	-0.2145	Rn.202968	Alb	albumin
1367556_s_at	0.0744	0.0000	0.0336	0.0187	0.0483	-0.0061	-0.2153	-0.0195	-0.1422	Rn.202968	Alb	albumin
1370214_at	-0.0495	-0.0906	0.0000	0.3272	0.2257	-0.0249	-0.0294	0.0994	0.0438	Rn.2005	Pvalb	parvalbumin

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